TECHNICAL NOTE

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The Use of the D-, L- Aspartic Ratio in Decalcified Collagen from Human Dentin as an Estimator of Human Age*

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ABSTRACT: Among the methods dealing with the age estimation, the evaluation of the ratio of the D-, L- form of the aspartic acid in tissues with a low metabolic turnover is considered to be the most precise. We introduced demineralization of the dentin with 0.5 M EDTA adjusted to pH = 7.4. The advantage of such a procedure is that after demineralization we obtained pure insoluble protein (collagen) and soluble noncollagenous proteins in one step. In this study we analyzed insoluble collagen. The amino acids obtained after the hydrolysis were derivatized into TFA isopropyl esters and analyzed by gas chromatography on Chirasil L-Val capillary column. We analyzed human dentin from the lower canines. The correlation coefficient was 0.93 for our set of 71 persons. The result concurred with those of other scientists.

KEYWORDS: forensic science, forensic odontology, forensic anthropology, human age estimation, chiral separation, aspartic acid

The estimation of human age is a topical problem in those forensic cases when the identity of an unknown dead body must be determined. Using teeth, two methods can be used for age estimation. The first one is an assessment of the morphological parameters, introduced by Gustafson many years ago which has been revised by several authors. The second one is the chemical method proposed by Bada et al. (1,2) where the age estimation is based on the evaluation of the ratio of the D- and L- forms of aspartic acid. This method has been studied intensively during the last ten years by many scientists. The opinion with this method ranges from very good (3–5) to moderate (6–8). Nevertheless, the age estimation according to the ratio of D/L enantiomers is considered as very precise and reliable, but the analysis of the dentin is quite sophisticated and includes several steps which may affect its accuracy. These steps include: 1) dentine sample preparation, 2) demineralization of the dentine and hydrolysis of protein, 3) desalting of the hydrolysate, 4) derivatization of free amino acids, 5) chiral separation of the amino acids derivatives by gas chromatography (GC) or high pressure liquid chromatography (HPLC). Each of these steps can be performed in different ways.

We have introduced a simple method of demineralization of the dentin using 0.5 M disodium salt of ethylenediaminetetraacetic acid (EDTA) because the resulting pure protein can be easily processed. EDTA binds calcium and magnesium into firm complexes at alkaline pH. This procedure has been used in the research of dentinal proteins (9–13) but had not been used before for age estimation based on the chiral analysis of the aspartic acid. The advantage of such sample processing is that the insoluble organic matrix (collagen) and soluble noncollagenous proteins (NCP) are obtained in one step.

Materials

We have examined intact lower canines from 71 (16 women, 55 men) persons between the ages of 15 and 95 years. Lower canines were selected because they are the longest present in the jaw. The teeth had been extracted from dead bodies and time since death did not exceed 72 h. The soft tissues were mechanically removed and tooth crown was cut off using forceps, then the teeth were rinsed with cool distilled water and stored at −25°C until processing.

Methods

Sample Preparation

Small chips of the primary dentin from the root tightly below dentino-enamel junction had been prepared for analysis. In this study we analyzed the dentin from the vestibular side of the root. The chips were cut off from the root in longitudinal direction using dental laboratory diamond disk cooled with water and then cleaned according to Othani et al. (4). The chips of dentin were precrushed into small shards and then powdered in the vibratory mill for 15 s.

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Demineralization

Fine powdered dentin was demineralized with 0.5 M Na_2EDTA which pH was adjusted to pH 7.4 with 1 M NaOH and stabilized with 0.05 mM sodium azide. Two milliliters of demineralizing solution were added to 5 mg of dentin powder in microvials and the samples were shaken intensively (1000 RPM) for 2 h at the ambient temperature. Afterwards, the samples were centrifuged at 1500 RPM, the supernatant discarded and the sediment—insoluble fraction, i.e., dentin collagen, washed and centrifuged three times to remove residues of Ca-, Mg-EDTA complexes and free EDTA. Approximately 1 mg of the dentine collagen was obtained using this procedure.

Hydrolysis

Protein was transfered into hydrolyzing tubes made of Simax™ glass (10 cm × 0.6 cm of internal diameter). Three hundred μL of 6 M hydrochloric acid was added, the tubes were evacuated, sealed with flame and heated at 100°C for 6 h. After hydrolysis, the hydrochloric acid was evaporated at 60°C under reduced pressure.

Amino Acid Analysis

Amino acids were derivatized according to Othani (4) into isopropyl esters using solution of isopropanol and acetyl chloride (4:1 v/v) at 100°C for 30 min, then the derivatizing solution was removed with a gentle stream of nitrogen and dry samples were derivatized with trifluoroacetic acid anhydride (TFAA) for 15 min at 60°C. Excess of TFAA was removed by a stream of nitrogen. Amino acid derivatives obtained were dissolved in 100 μL methylacetate and analyzed by gas chromatography on the chiral XE-60-S-VAL-SA-PEA fused silica column (50 m, 0.25 mm ID, 0.12 μm film thickness) purchased from Chrompack. The gas chromatograph FISONS 8160 equipped with autosampler AS800 and flame ionization detector, was used for sample analysis. Each sample was chromatographed four times. Sets of D- and L- standards of pure amino acids were processed in the same way as the samples to evaluate their retention times.

Gas Chromatography Separation Conditions

The following experimental conditions were used for the separation of aminoacid enantiomers. The carrier gas flow rate was 1 mL/min of hydrogene. The temperature program: 75°C for 10 min, 3°C/min to 95°C, then 5°C/min to 175°C, 10 min. The temperature of the injector was 230°C and of the detector 250°C. The sample split ratio was 1/80 and the injected sample volume 1 to 3 μL.

Statistical Processing

The obtained data have been processed using software Microsoft Excel and "Statistica for WIN v. 5.1" (StartSoft, inc, USA).

Results

Amino Acid Spectrum

Figure 1 shows typical chromatogram of the mixture of D- and L-amino acid standards. The experimental separation conditions used on this capillary column gave very good separation of D- and L-amino acid enamtiomers. Figure 2 represents an amino acid spectrum typical for protein samples of dentin collagen. The large peaks of the glycine, proline, and hydroxyproline proves the dentinal collagen. Figure 3 shows excellent separation of D- and L- aspartic acid

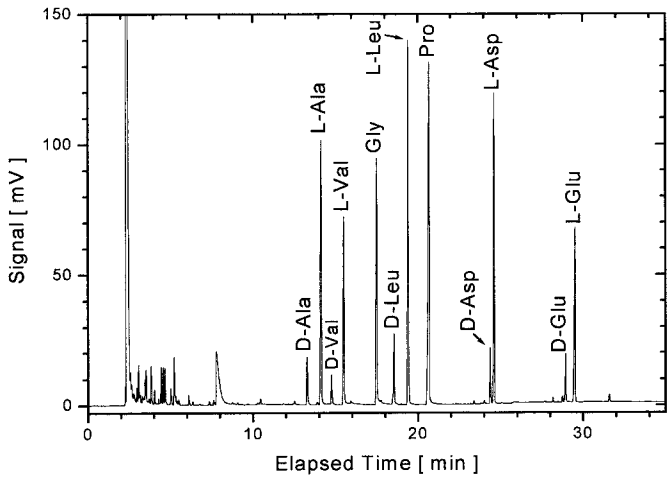

FIG. 1—*GC analysis of amino acid enantiomer standards (TFA-isopropyl esters derivatives).*

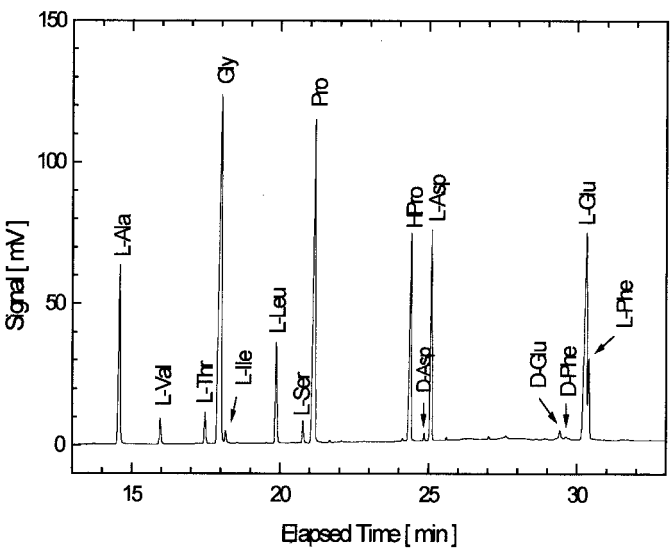

FIG. 2—*GC analysis of amino acid enantiomers (TFA-isopropyl esters derivatives) after hydrolysis of dentin collagen.*

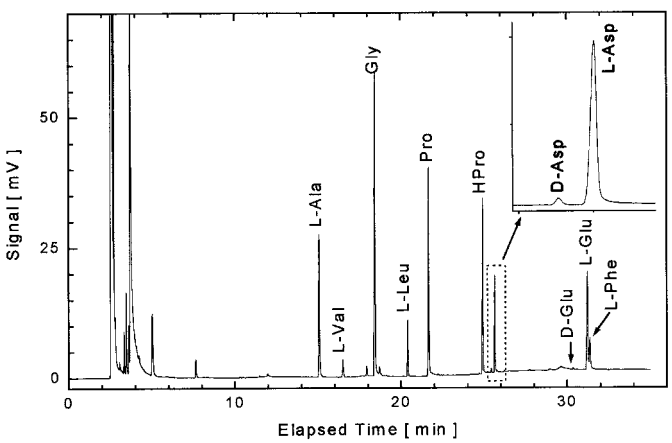

FIG. 3—*GC analysis of amino acid enantiomers (TFA-isopropyl esters derivatives) after hydrolysis of dentin collagen.*

which was achieved in all chromatograms demonstrating a good reproducibility of the sample treatment procedure. Small peaks of D-serine, D-glutamic acid and D-phenylalanine have been also detected but their amount did not correlate with the age.

Statistical Data Evaluation

The coefficient of racemization (*KR*) is commonly used and it is expressed by the equation:

$$KR = \ln\left[\frac{1 + D/L}{1 - D/L}\right]$$

Characters *D* and *L* represent peak areas of the relating enantiomer. Correlation coefficient (*R*) and regression equation was calculated for whole data sets and for reduced data sets where data of old persons has been excluded. Figures 4 and 5 show the plot of obtained data.

The regression equation for the whole data set of 71 persons between the age of 15 and 95 years is:

$$KR = 0.0006 \times \text{age} + 0.0352, \quad R = 0.93$$

The regression equation for the reduced data set of 46 persons between the age of 15 and 60 years is:

$$KR = 0.0009 \times \text{age} + 0.02269, \quad R = 0.96$$

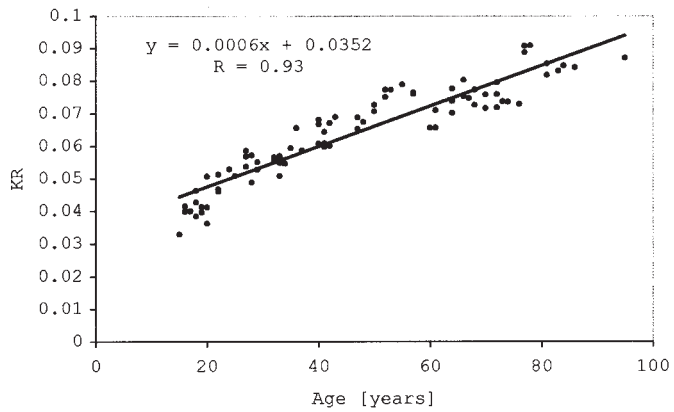

FIG. 4—*Extent of aspartic acid racemization—the plot of data for the whole set.*

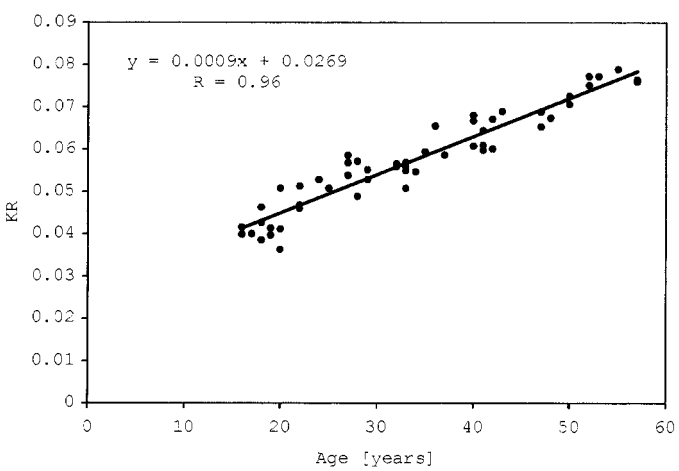

FIG. 5—*Extent of aspartic acid racemization—the plot of data for the set excluding persons older than 60 years.*

Discussion

In this study we have confirmed that estimation of the human age based on evaluation of the ratio of D- and L- forms of aspartic acid can be used. The results obtained for the analysis of the insoluble part of organic matrix of the dentin after demineralization with EDTA are in agreement with data reported by Ohtani et al. (3–4) and Ritz et al. (5,17).

Nevertheless the analysis of the dentin is demanding and has two critical points. Based on our previous results (14–15), these critical points are the hydrolysis and the chiral separation. As far as the hydrolysis the only correct method is the hydrolysis in evacuated flame-sealed glass tubes. Hydrolysis under the HCl reflux lasting 6 h and performed at 100°C did not give amino acids spectrum typical for collagen. Some scientists hydrolyzed the collagen samples in the screw-top vials. We have found this procedure unsuitable because it is not possible to guarantee the vial tightness and therefore the sample often got dried during the heating, leading to artificial racemization causing invalid results. Another disadvantage of this method is an irreproducible hydrolysis demonstrated by an incomplete amino acid spectrum in chromatograms.

The chiral separation is the second critical point of the sample treatment. Good separation is the basic condition, because we are working with very small amounts of the D- form and bad separation leads to mistakes during the peak integration. The sample volume injected on GC should be modified in a such way that the resulting peak area of D- aspartic acid is reproducibly integrated. The relative standard deviation of peak area of D-aspartic acid should be less than 5%. The variation of this peak area decreases the correlation between *KR* and age dramatically. At very low content of D- aspartic acid, small overloading of the sample column capacity, which causes fronting of large peaks is possible and does not disturb the separation of enantiomers because of sufficient separation coefficient.

The samples of dentin collagen processed with the described method gave reliable results. The error of the estimation was calculated from the coefficients for lower and upper border of confidence at 95% and it was ± 4 years for the data until 60 years. The reproducibility of the analytical procedure has been tested on 14 randomly selected samples. These samples were repeatedly processed in the same way. The obtained values were tested using Student pair *t*-test. No statistically significant differences were found neither at 5% nor at 1% level of significance [*t* crit (1) = 1.7; *t* crit (2) = 2.05; *t* stat = 0.35)]. Correlation coefficient between both series was $R = 0.906$. The plot of *Kr* to *KR* repeated values (Youden method) indicates a small systematic proportional error. Of course, results are also fraught with random error.

The correlation coefficient of age between *KR* is 0.93 for the whole set of data. It can be seen from the plot of the data that the ratio of D- and L- form of aspartic acid rises with increasing age. Nevertheless, according to our set of data it seems that the increment of *KR* in higher age groups is somewhat slower than compared with people until 60 years of age. We suppose that this fact, found in our data, does not decrease the significance of age estimation using an assessment of *KR* of aspartic acid. The values from our set obtained by linear approximation are more reliable in the area of young and middle age groups where the method should be employed. Owing to the fact that few persons above the age of 60 still have some teeth suitable for the analysis, the risk of distortion of conclusions is small. Indicated data represents a selection of existing population and therefore conclusions based on obtained data will represent this set better than the set enriched with data taken

from persons older than 60 years. The values of *KR* in the area above the 60 years of age cover up men (=17) and women (=8). Small number of women in the set was the reason why the influence of the sex on the *KR* was not studied. Even that there are not enough values for men and women separately, it seems that sex has not effect the differences of *KR* values.

Conclusion

The age assessment, using the evaluation of the D/L ratio of the aspartic acid is optional, quite precise method that can be used in the forensic practice. We recommend to perform parallel analyses of known and unknown samples when the age should be estimated by this method.

Acknowledgments

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