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TECHNICAL NOTE

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Technical notes for age estimation using the femur: influence of various analytical conditions on D-aspartic acid contents

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Abstract In order to make a better estimate of the individual age using bone samples, we evaluated the effects of various criteria on the analytical measurement of D-aspartic acid contents. Using compact bone from a male femur, we varied six analytical conditions (sample volume, sample particle size, hydrolysis temperature, hydrolysis time, hydrochloric acid volume during hydrolysis, and hydrochloric acid concentration during hydrolysis). D-form/L-form ratios were affected most by hydrolysis temperature (estimated age differences were 3.03 years/°C), followed in order by hydrochloric acid volume (1.44 years/ml) and hydrochloric acid concentration (0.69 years/0.1 M). Larger sample particle sizes and hydrochloric acid volumes during hydrolysis tended to result in lower racemization rates. Nonetheless, within a range of 5–50 mg, sampling volume did not affect the detected D-aspartic acid contents. Since the racemization reaction rate in femur compact bone is slower than in dentin, bone samples seem to be more greatly influenced by analytic conditions than dentin. Tests must therefore be performed with caution, especially with regard to the hydrolysis temperature, hydrochloric acid volume and concentration when estimating age using femur samples.

Keywords Age determination · Femur · Racemization · D-aspartic acid · Total amino acid

Introduction

The amino acids found in vital tissues are commonly L-amino acids [1]. However, in tissues with slow metabolism such as teeth [2, 3, 4, 5, 6, 7, 8, 9, 10, 11], the lens of the eye [12, 13], myelin proteins in the brain [14] and compact bone and cartilage [15, 16, 17, 18, 19, 20, 21, 22], D-amino acids accumulate with age as a result of racemization. In particular, since the level of D-aspartic acid increases at a steady rate in dentin, this tissue is commonly used for age estimation [23].

Common D-amino acids present in bones are, in the following order of concentration, aspartic acid>alanine=glutamic acid>isoleucine=leucine. Levels of these D-amino acids are used to estimate the age of fossilized bones [1, 24]. Ritz and colleagues [16, 19] documented an extremely high correlation between age and aspartic acid racemization in osteocalcin of the human skull (coefficient of correlation, r=0.99). Pfeiffer and colleagues [17, 18] examined two different fractions of rib cartilages and compact bones, namely an acid-soluble peptide fraction and an acid-insoluble collagen-rich fraction, and reported that the coefficients of correlation between aspartic acid racemization and age ranged from r=0.72 to r=0.97.

We have previously analyzed the relationship between age and aspartic acid racemization in the total amino acid content of the femur, and found a gender difference where the rate of aspartic acid racemization was greater in males [20]. Furthermore, we reported that the coefficient of correlation between age and aspartic acid racemization of seven different bones was highest in the femur [22].

Therefore, we investigated the effects of the following six parameters on detectable D-aspartic acid in the total amino acid content of a male femur: sample volume, sample particle size, hydrolysis temperature, hydrolysis time, hydrochloric acid volume during hydrolysis, and hydrochloric acid concentration during hydrolysis. Although it is already known that some of these factors have an effect on D/L ratios [11, 25, 26, 27], we further investigated how much they affect the age estimation.

Materials and methods

A femur was collected from a deceased 48-year-old man within 1 year of his death. The femur was fixed in 10% formaldehyde, then pulverized into powder with a particle size range of 74~297 μm as follows: the bone was sectioned using an autopsy saw, and

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divided into fragments of about 1 cm^2 using a low-speed saw (Isomet 11-1180 low-speed saw, Buhler, Chicago, Ill.). The surface of these bone fragments was then polished using a grindstone to remove soft tissue and cancellous bone and to recover compact bone. The polished bone fragments were washed by ultrasound in distilled water 3 times, in ethanol once and in ether once for 5 min each wash. The washed bone fragments were dried, pulverized using a mill (Fritsch, Idav-oberstein, Germany), and divided into four particle sizes using a sieve shaker (M-2, Tsutsui Rikagaku, Tokyo). The racemization (D/L ratio) of aspartic acid was then measured from the total amino acid content of the femur particles. D- and L-forms were separated by gas chromatography (GC-17A, Shimadzu, Kyoto) using an FS capillary column coated with chirasil-val (25 m in length, 0.3 mm in diameter, GL Science, Tokyo). Aspartic acid D/L ratios were calculated by measuring the peak area of D-aspartic acid and L-aspartic acid and applying the equation ln[(1+D/L)/(1-D/L)].

Each of the six test-related variables was divided into four levels as follows: sample volume (5, 10, 20, 30, 40 and 50 mg), sample particle size (<53, 53–74, 74–105, 105–177, 177–297 and >297 μm), hydrolysis temperature (90, 95, 100, 105, 110 and 120°C), hydrolysis time (6, 24, 36, 48, 60 and 72 h), hydrochloric acid volume during hydrolysis (5, 8, 10, 12, 15 and 20 ml), and hydrochloric acid concentration during hydrolysis (1.5, 2, 3, 4, 5 and 6 M). Except for the test-related conditions, the standard experimental conditions were as follows: sample volume 10 mg, sample particle size 74–297 μm, hydrolysis temperature 100°C, hydrolysis time 6 h, hydrochloric acid volume during hydrolysis 5 ml, and hydrochloric acid concentration during hydrolysis 6 M [22].

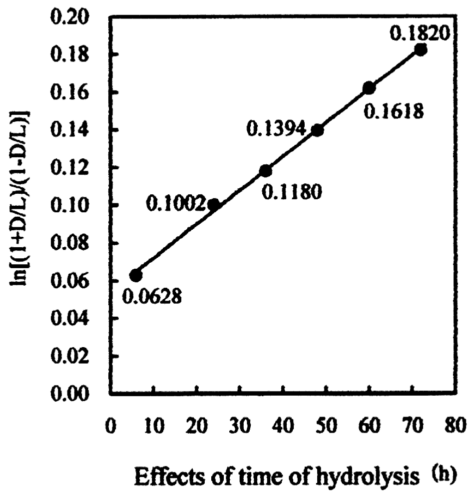

Fig. 2 Changes in racemization rates in relation to changes in hydrolysis time

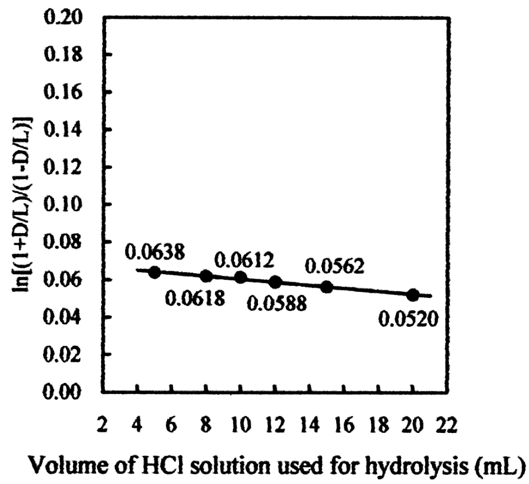

Fig. 3 Changes in racemization rates in relation to changes in hydrochloric acid volume during hydrolysis

Results

Excluding sampling volume, the levels of the other five test-related variables fluctuated linearly in the first order, and demonstrated a high degree of correlation between test-related parameters and racemization rates ($r \geq 0.977$).

We have previously reported the racemization reaction rate constant in the femur to be [$k(y)=2.79\times10^{-4}$] [22]. Using this constant, we deduced the value of k from the racemization rate equation for each of the six test-related parameters, and then estimated age based on the k value for the femur. Age estimation was affected most by hydrolysis temperature (3.03 years/°C), followed by hy-

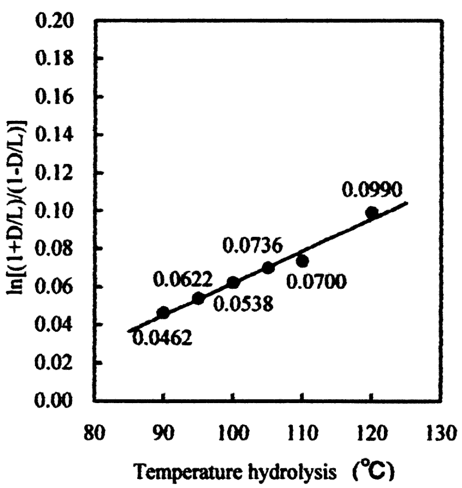

Fig. 1 Changes in racemization rates in relation to changes in hydrolysis temperature

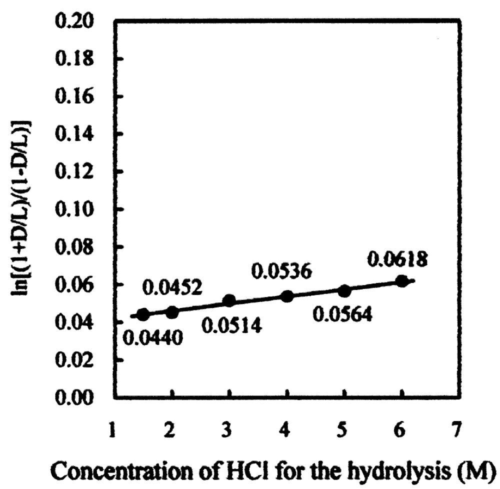

Fig. 4 Changes in racemization rates in relation to changes in hydrochloric acid concentration during hydrolysis

drochloric acid volume (1.44 years/ml) and hydrochloric acid concentration (0.69 years/0.1 M), in that order. Within a range of 5–50 mg, the sample volume did not alter the D/L ratios. In addition, a tendency was observed for larger sample particle sizes and hydrochloric acid volumes during hydrolysis, to exhibit lower racemization rates (Figs. 1, 2, 3, 4).

Discussion

In recent years, several reports have been published regarding age estimation based on aspartic acid racemization in bones and cartilages [15, 16, 17, 18, 19, 20, 21, 22]. We previously reported that of seven types of bones and a cartilage analyzed, the degree of correlation between age and aspartic acid racemization in the total amino acid content was highest in the male femur [22]. We therefore decided to investigate the effects of six test-related variables on age estimation based on aspartic acid racemization in the total amino acid content of pulverized male femur.

Higher hydrolysis temperatures tended to be associated with greater D/L ratios. In most chemical reactions, higher reaction temperatures tend to result in more active molecules, and thus faster reactions. In addition, longer reaction times tended to be associated with increased D/L ratios. Conversely, larger hydrochloric acid volumes tended to be associated with decreased D/L ratios because the sample concentration decreased when more hydrochloric acid was added. Moreover, higher concentrations of hydrochloric acid tended to be associated with faster reactions, and thus greater D/L ratios. As far as the sample particle size was concerned, smaller particle sizes tended to be associated with greater D/L ratios. The reason for this is that although the sample volume remains unchanged, more particles are present when the sample particle size is small. This is also the case with teeth. However, within a range of 5–50 mg, sample volume did not alter the racemization rate. Among the six test-related parameters, hydrolysis temperature, hydrochloric acid volume and hydrochloric acid concentration more seriously affected the age estimation.

The effect of each test-related parameter on the D/L ratios in femur compact bone showed a similar tendency to that in dentin [7]. However, when age was estimated based on the racemization reaction rate constants, the effect of hydrolysis temperature on age estimation in femur cortical bone was about twice that in dentin, while the effect of hydrochloric acid volume on age estimation in femur cortical bone was about 2.5 times that in dentin. This was because the k value for dentin is about 2.3 times that for femur cortical bone. This difference in racemization rate constant between femur cortical bone [22] and dentin [7] is believed to be attributable to biological environmental differences. As a result, slight differences in testing procedures magnify the estimated ages. Caution must therefore be exercised when estimating age based on aspartic acid racemization in bones.

Ritz and colleagues [16, 19] documented an extremely high degree of correlation between age and the level of D-aspartic acid in osteocalcin, which is the most abundant non-collagenous protein in the skull. Since we believe that levels of non-collagenous bone proteins and osteocalcin can easily vary depending on techniques used, age estimation based on aspartic acid racemization in bones can be easily and rapidly performed with a reliable reproducibility and stability when assessing racemization in the total amino acid content. Consequently, when estimating age using a bone sample, it is necessary to simultaneously analyze a sufficiently large number of bone samples of known age paying special attention to the analytical conditions.

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