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# Comparison of Age Estimated from Degree of Racemization of Aspartic Acid, Glutamic Acid and Alanine in the Femur

**ABSTRACT:** Aspartic acid (Asp) is generally used for estimation of age by measuring the degree of racemization. For other amino acids, however, there are few reports regarding the usefulness of the degree of racemization for the estimation of age. Accordingly, in this study using the femur (obtained from 21 cadavers) as the specimen, we measured the degree of racemization of glutamic acid (Glu) and alanine (Ala) along with Asp in the total amino acid (TAA) fraction as well as in acid-insoluble collagen-rich (IC) and acid-soluble peptide (SP) sub-fractions. We compared the degrees of racemization of each amino acid and the accuracy of the ages estimated from them. The degree of racemization and the reaction rate of racemization were ranked in the order of Asp>Glu>Ala in the TAA and IC fractions, but Asp>Ala>Glu in the SP fraction. It is noteworthy that the degrees of racemization differed between the three amino acids depending on the fraction tested. The correlation coefficient (r) between the degree of racemization and the chronological age was higher in the SP than in the TAA or IC fraction. Among three amino acids, Asp showed the highest correlation coefficient as predicted. The present study confirmed that Asp from the SP fraction is the best indicator for age estimation using racemization rates.

**KEYWORDS:** forensic science, age estimation, femur, D-aspartic acid, D-glutamic acid, D-alanine

Racemization is facilitated in tissues where metabolism proceeds slowly. Metabolic turnover in bone is more active than in teeth, due to the continuous formation and resorption of bone. Ritz et al. (1,2) obtained an acid-soluble peptide fraction from the skull, and reported that D-Asp in osteocalcin increased remarkably with age. Pfeiffer et al. (3,4) divided the TAA fraction obtained from rib cartilage and cortical bone into IC and SP fractions according to our method (5), and found a high correlation between age and the degree of racemization of Asp. In addition, we (6) reported a sex difference in the degree of racemization of Asp in bone.

There have to date been many reports of the application of measurement of racemization of amino acids to estimation of the dates of fossil bones (7–11). In most of these studies, Asp was used as the indicator for estimation of the date, although racemization of other amino acids was used in some studies (12,13). Due to the small amounts of D-Glu and D-Ala and technical difficulty to separate three enantiomers completely, only a few reports have used Glu and Ala as indicators in addition to Asp. However, it is uncertain whether Glu and Ala are possible as an indicator for age estimation using racemization rates. In 1991 we (14) have succeeded in detection and complete separation of D-Glu and D-Ala in TAA from teeth. In the present study, we measured the degrees of racemization of Glu and Ala as well as Asp in TAA and its IC and SP fractions from the femur, and compared their applicability to estimation of age.

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## Materials and Methods

### *Collection of the Specimen*

Specimens were obtained from males aged 16–79 years. Each specimen consisted of the compact material of the femur of 21 cadavers donated after death and fixed in formalin. The femur was transected at its middle portion with a cutter and a slice of bone was removed. The transected surface was polished on a grindstone, and the compact protein was isolated by removing flesh and spongy material. They were cut into pieces of 4 $mm^2$ in size, and were rinsed successively in distilled water, ethanol and ether for 5 min, respectively, in an ultrasonic bath, then dried and pulverized in a porcelain mortar. The powdered material was passed through a sieve, and particles of 74–297 μm in size were used for analysis. Ten mg of powder was used for measuring racemization in the TAA fraction. To divide the material into the IC and SP fractions, 100 mg of powder was soaked in 1 mL of 6 M hydrochloric acid and centrifuged at 4000× g at 5°C for 1 h; the precipitate and supernatant were used as the IC and SP fractions, respectively, after being dried in an evaporator.

We analyzed the amounts of the D- and L-isomers of Asp using the method previously reported in our paper (15), and calculated the degree of racemization, i.e., the D/L ratio and $\ln[(1 + D/L)/(1 - D/L)]$.

### *Computation of Equation for the Reaction Rate of Racemization*

The equation for the reaction rate of racemization was obtained from the chronological age and the D/L ratio. Against the chronological age on the X axis, the D/L ratio expressed as $\ln[(1 + D/L)/(1 - D/L)]$ was plotted on the Y axis. The equation fitting the regression line obtained by the least square method was calculated from the following equation:

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

where $\ln[(1 + D/L)/(1 - D/L)]$ denotes a logarithmic transform of the D/L ratio, t the age, and k the reaction rate constant of racemization.

The equation for estimation of age was obtained by a coordinate transformation of the equation of the reaction rate of racemization, i.e., by plotting age on the Y axis and the D/L ratio expressed as $\ln[(1 + D/L)/(1 - D/L)]$ on the X axis; the equation fitting the regression line obtained by the least square method was calculated for the estimation of age from the D/L ratio.

## Results and Discussion

Recently, a number of studies on the estimation of age using the racemization method have been reported (1,6,17). One of the crucial points in such studies is to set up conditions which allow complete separation between the D- and L-isomers, showing them as distinct sharp peaks on a gas chromatogram. As shown in Fig. 1 illustrating the gas chromatogram, the D- and L-isomers of Asp, Glu and Ala are clearly separated from one another. As regards the constituent amino acids, it can be seen that hydroxyproline is present at high levels in the TAA and IC fractions, showing collagen to be the main protein component of bone. In contrast, only a small amount of hydroxyproline is contained in the SP fraction, suggesting that this contains a large amount of non-collagen protein. In a study of the estimation of age using the TAA, IC and SP fractions from femurs of known age, we reported that the correlation between the estimated and chronological ages was higher in males than in females (6). In males, the highest correlation coefficient between the age estimated with TAA and the chronological age was obtained from the femur, when the skull, sternum, rib cartilage, lumbar vertebra, coxal bone, sacral vertebra and femur were tested (18). We also reported that a large amount of the D-isomer of amino acids could be detected from the TAA and IC fractions (5). It has been reported, however, that the amount of D-amino acids in the SP fraction varies with the amount as well as the type of acid used for extraction, the particle size of the material and the centrifugal force used (6). Needless to say, detection of amino acids from the SP fraction requires careful and consistent analytical procedures.

We extracted TAA from the male femur, divided it into the IC and SP fractions, and compared the racemization rate constants and correlation coefficients between Glu and Ala in addition to Asp (Tables 1–3).

The degree of racemization in the TAA and IC fractions exhibited the order Asp>Glu>Ala (Figs. 2 and 3), while in the SP fraction the order was Asp>Ala>Glu (Fig. 4). It is noteworthy that the degree of racemization of Ala was higher than Glu in the SP fraction. This difference may be due to a significant difference in protein constituents.

The reaction rate of racemization was in the order of Asp> Glu>Ala in the TAA and IC fractions and Asp>Ala>Glu in the SP fraction, which was the same order as the degree of racemization (Figs. 2–4). It has been reported that the reaction rate of racemization in bovine bone showed the order of Asp > Ala = Glu > isoleucine ≅ leucine (12).

The correlation coefficient (r) between the degree of racemization of the three types of amino acid and the chronological age was obtained from the equation fitting the regression line. It was found that the value of r obtained from the SP fraction was higher than that obtained from the TAA or IC fractions. As predicted, the correlation

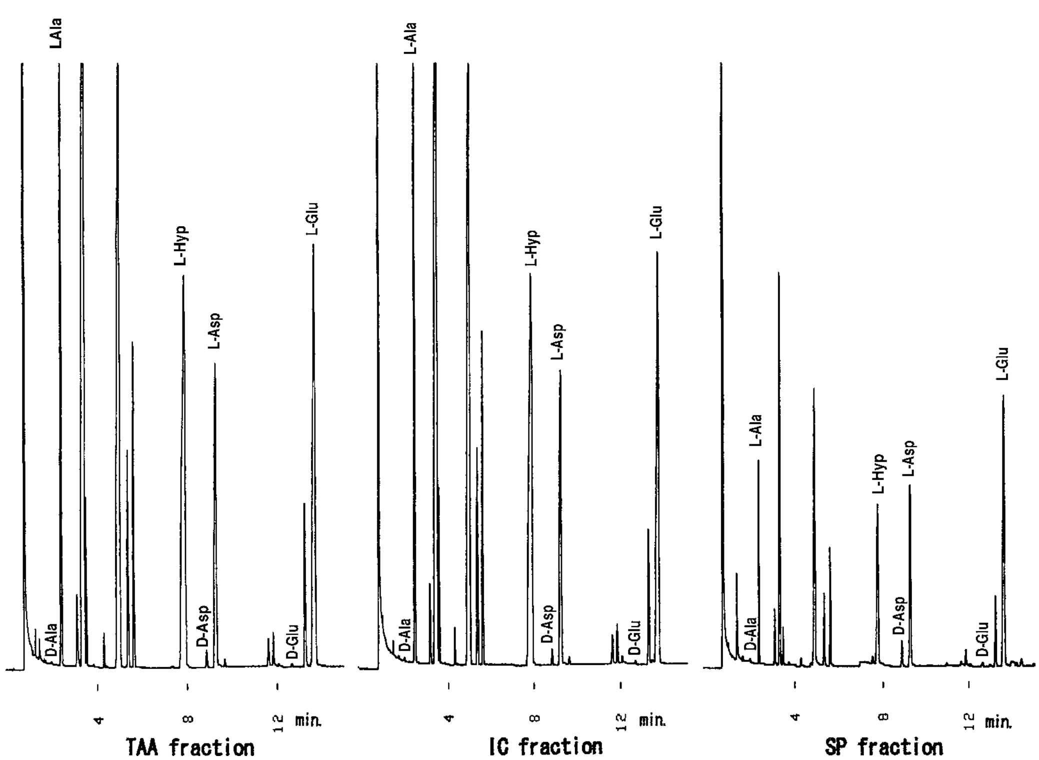


FIG. 1—*Gas chromatogram of N-trifluoroacetyl isopropyl esters of amino acid in femur. Column temperature, 90°C, 1 min hold, and then programmed to 180°C at 4°C/min; injection temperature. 250°C; carrier gas, helium; split ratio, 40:1. TAA is the total amino acid fraction, IC is the acid-insoluble collagen-rich fraction, and SP is the acid-soluble peptide fraction.*

TABLE 1—*Degree of racemization of three amino acids in the total amino acid fraction from the femur and the estimated ages.*

| Individual No. | Chronological Age | Asp D/L (X) | Asp Estimated Age (Y) | Glu D/L (X) | Glu Estimated Age (Y) | Ala D/L (X) | Ala Estimated Age (Y) |
|---|---|---|---|---|---|---|---|
| 1 | 16 | 0.0660 | 17 | 0.0092 | 32 | 0.0060 | 19 |
| 2 | 30 | 0.0768 | 32 | 0.0100 | 45 | 0.0068 | 48 |
| 3 | 47 | 0.0864 | 46 | 0.0104 | 52 | 0.0068 | 48 |
| 4 | 47 | 0.0850 | 44 | 0.0102 | 48 | 0.0072 | 62 |
| 5 | 49 | 0.0860 | 45 | 0.0098 | 42 | 0.0070 | 55 |
| 6 | 53 | 0.0936 | 56 | 0.0102 | 48 | 0.0068 | 48 |
| 7 | 55 | 0.0934 | 56 | 0.0110 | 61 | 0.0070 | 55 |
| 8 | 57 | 0.0914 | 53 | 0.0102 | 48 | 0.0068 | 48 |
| 9 | 58 | 0.0944 | 57 | 0.0102 | 48 | 0.0072 | 62 |
| 10 | 59 | 0.0996 | 65 | 0.0110 | 61 | 0.0072 | 62 |
| 11 | 61 | 0.1012 | 67 | 0.0112 | 64 | 0.0074 | 69 |
| 12 | 62 | 0.0966 | 60 | 0.0106 | 55 | 0.0070 | 55 |
| 13 | 63 | 0.0994 | 64 | 0.0120 | 77 | 0.0072 | 62 |
| 14 | 67 | 0.0996 | 65 | 0.0108 | 58 | 0.0070 | 55 |
| 15 | 67 | 0.1016 | 67 | 0.0106 | 55 | 0.0072 | 62 |
| 16 | 70 | 0.1026 | 69 | 0.0112 | 64 | 0.0070 | 55 |
| 17 | 70 | 0.1040 | 71 | 0.0114 | 68 | 0.0074 | 69 |
| 18 | 72 | 0.1004 | 66 | 0.0120 | 77 | 0.0076 | 77 |
| 19 | 74 | 0.1046 | 72 | 0.0118 | 74 | 0.0074 | 69 |
| 20 | 77 | 0.1062 | 74 | 0.0112 | 64 | 0.0072 | 62 |
| 21 | 79 | 0.1100 | 79 | 0.0120 | 77 | 0.0076 | 77 |
| Coefficient of correlation | | 0.980 | | 0.821 | | 0.822 | |
| Formula to estimate age | | Y = 1424.05X−76.83 | | Y = 16079.85X−115.10 | | Y = 35864.49X−195.41 | |

TABLE 2—*Degree of racemization of three amino acids in the acid-insoluble collagen-rich fraction from the femur and the estimated ages.*

| Individual No. | Chronological Age | Asp D/L (X) | Asp Estimated Age (Y) | Glu D/L (X) | Glu Estimated Age (Y) | Ala D/L (X) | Ala Estimated Age (Y) |
|---|---|---|---|---|---|---|---|
| 1 | 16 | 0.0608 | 17 | 0.0088 | 32 | 0.0058 | 28 |
| 2 | 30 | 0.0674 | 29 | 0.0092 | 38 | 0.0062 | 42 |
| 3 | 47 | 0.0758 | 44 | 0.0100 | 51 | 0.0064 | 49 |
| 4 | 47 | 0.0820 | 55 | 0.0098 | 48 | 0.0062 | 42 |
| 5 | 49 | 0.0788 | 49 | 0.0092 | 38 | 0.0066 | 56 |
| 6 | 53 | 0.0848 | 60 | 0.0100 | 51 | 0.0068 | 63 |
| 7 | 55 | 0.0832 | 57 | 0.0106 | 61 | 0.0068 | 63 |
| 8 | 57 | 0.0824 | 56 | 0.0098 | 48 | 0.0064 | 49 |
| 9 | 58 | 0.0828 | 57 | 0.0098 | 48 | 0.0070 | 70 |
| 10 | 59 | 0.0832 | 57 | 0.0106 | 61 | 0.0066 | 56 |
| 11 | 61 | 0.0826 | 56 | 0.0108 | 64 | 0.0070 | 70 |
| 12 | 62 | 0.0838 | 58 | 0.0104 | 58 | 0.0066 | 56 |
| 13 | 63 | 0.0874 | 65 | 0.0110 | 67 | 0.0070 | 70 |
| 14 | 67 | 0.0864 | 63 | 0.0106 | 61 | 0.0068 | 63 |
| 15 | 67 | 0.0870 | 64 | 0.0102 | 55 | 0.0066 | 56 |
| 16 | 70 | 0.0860 | 62 | 0.0112 | 71 | 0.0070 | 70 |
| 17 | 70 | 0.0910 | 72 | 0.0112 | 71 | 0.0070 | 70 |
| 18 | 72 | 0.0912 | 72 | 0.0118 | 80 | 0.0064 | 49 |
| 19 | 74 | 0.0932 | 76 | 0.0114 | 74 | 0.0070 | 70 |
| 20 | 77 | 0.0916 | 73 | 0.0110 | 67 | 0.0068 | 63 |
| 21 | 79 | 0.0956 | 80 | 0.0116 | 77 | 0.0070 | 70 |
| Coefficient of correlation | | 0.969 | | 0.877 | | 0.773 | |
| Formula to estimate age | | Y = 1815.53X−93.18 | | Y = 16242.62X−110.67 | | Y = 35029.41X−174.82 | |

TABLE 3—*Degree of racemization of three amino acids in the acid-soluble peptide fraction from the femur and the estimated ages.*

| | | Asp | | Glu | | Ala | |
|---|---|---|---|---|---|---|---|
| Individual No. | Chronological Age | D/L (X) | Estimated Age (Y) | D/L (X) | Estimated Age (Y) | D/L (X) | Estimated Age (Y) |
| 1 | 16 | 0.0808 | 14 | 0.0144 | 27 | 0.0194 | 19 |
| 2 | 30 | 0.1366 | 34 | 0.0172 | 37 | 0.0254 | 32 |
| 3 | 47 | 0.1722 | 47 | 0.0204 | 49 | 0.0384 | 61 |
| 4 | 47 | 0.1698 | 46 | 0.0186 | 42 | 0.0308 | 44 |
| 5 | 49 | 0.1778 | 49 | 0.0204 | 49 | 0.0324 | 47 |
| 6 | 53 | 0.1760 | 48 | 0.0188 | 43 | 0.0376 | 59 |
| 7 | 55 | 0.1990 | 56 | 0.0236 | 60 | 0.0380 | 60 |
| 8 | 57 | 0.1952 | 55 | 0.0198 | 47 | 0.0382 | 60 |
| 9 | 58 | 0.2160 | 63 | 0.0264 | 70 | 0.0414 | 67 |
| 10 | 59 | 0.2046 | 59 | 0.0254 | 66 | 0.0386 | 61 |
| 11 | 61 | 0.2144 | 62 | 0.0230 | 58 | 0.0352 | 54 |
| 12 | 62 | 0.2070 | 59 | 0.0224 | 56 | 0.0362 | 56 |
| 13 | 63 | 0.2206 | 64 | 0.0254 | 66 | 0.0410 | 66 |
| 14 | 67 | 0.2204 | 64 | 0.0236 | 60 | 0.0380 | 60 |
| 15 | 67 | 0.2218 | 65 | 0.0238 | 61 | 0.0356 | 54 |
| 16 | 70 | 0.2364 | 70 | 0.0264 | 70 | 0.0392 | 62 |
| 17 | 70 | 0.2396 | 71 | 0.0270 | 72 | 0.0436 | 72 |
| 18 | 72 | 0.2396 | 71 | 0.0256 | 67 | 0.0424 | 69 |
| 19 | 74 | 0.2404 | 71 | 0.0284 | 77 | 0.0442 | 73 |
| 20 | 77 | 0.2476 | 74 | 0.0258 | 68 | 0.0416 | 68 |
| 21 | 79 | 0.2640 | 80 | 0.0290 | 79 | 0.0468 | 79 |
| Coefficient of correlation | | 0.988 | | 0.897 | | 0.906 | |
| Formula to estimate age | | Y = 360.21X−14.70 | | Y = 3531.20X−22.91 | | Y = 2187.52X−22.95 | |

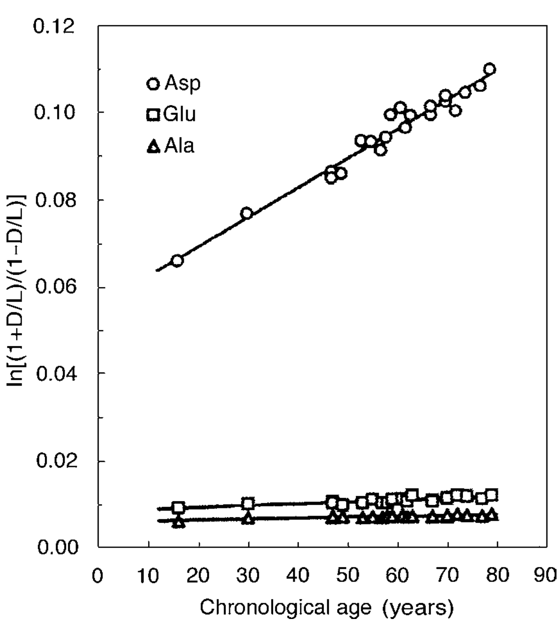


FIG. 2—*Correlation between the degree of racemization of total amino acids obtained from the femur and chronological age.*

Asp, $\ln[(1 + D/L)/(1 - D/L)] = 0.000674t + 0.0556; k = 3.3571 \times 10^{-4}$
Glu, $\ln[(1 + D/L)/(1 - D/L)] = 0.000042t + 0.0084; k = 2.0425 \times 10^{-5}$
Ala, $\ln[(1 + D/L)/(1 - D/L)] = 0.000019t + 0.0060; k = 9.4875 \times 10^{-6}$

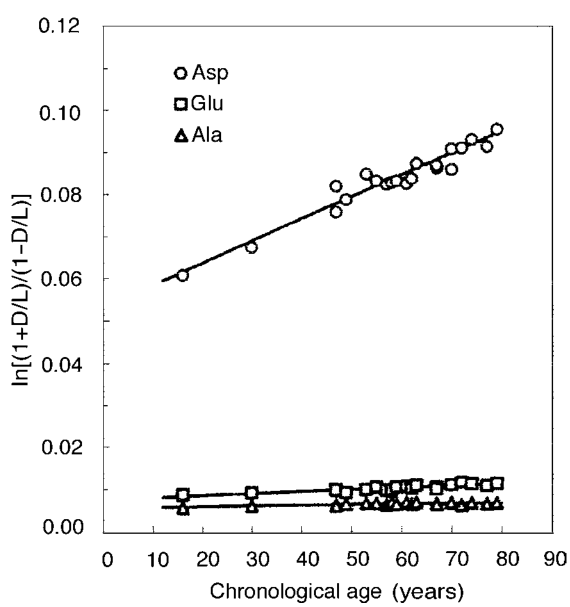


FIG. 3—*Correlation between the degree of racemization of amino acids in the acid-insoluble collagen-rich fraction (IC) obtained from the femur and chronological age.*

Asp, $\ln[(1 + D/L)/(1 - D/L)] = 0.000518t + 0.0533; k = 2.5880 \times 10^{-4}$
Glu, $\ln[(1 + D/L)/(1 - D/L)] = 0.000047t + 0.0077; k = 2.3651 \times 10^{-5}$
Ala, $\ln[(1 + D/L)/(1 - D/L)] = 0.000017t + 0.0057; k = 8.5370 \times 10^{-6}$

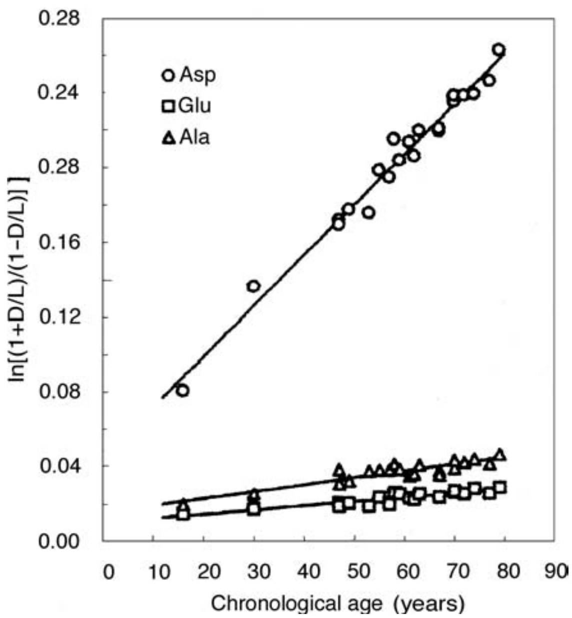


FIG. 4—*Correlation between the degree of racemization of amino acids in the acid-soluble peptide fraction (SP) obtained from the femur and chronological age.*

Asp, $\ln[(1 + D/L)/(1 - D/L)] = 0.002712t + 0.0446; k = 1.3558 \times 10^{-3}$
Glu, $\ln[(1 + D/L)/(1 - D/L)] = 0.000228t + 0.0097; k = 1.1398 \times 10^{-4}$
Ala, $\ln[(1 + D/L)/(1 - D/L)] = 0.000375t + 0.0153; k = 1.8762 \times 10^{-4}$

of the degree of racemization of Asp with the chronological age was highest among three amino acids which was in accord with our previous report (14) (Figs. 2–4).

Thus, the present work confirmed that Asp is the best indicator for age estimation using racemization rates in view of technical easiness and high correlation coefficient.

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