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Evaluation of Aspartic Acid Racemization Ratios in the Human Femur for Age Estimation

REFERENCE: Ohtani S, Matsushima Y, Kobayashi Y, Kishi K. Evaluation of aspartic acid racemization ratios in the human femur for age estimation. J Forensic Sci 1998;43(5):949–953.

ABSTRACT: Levels of *D*-aspartic acid (*D*/*L* ratio) in cranial non-collagen proteins (acid-soluble peptide fractions) have been reported to increase with age. We isolated total amino acid fractions from the femur and separately isolated acid-insoluble collagen fraction and acid-soluble peptide fractions; then *D*/*L* ratios were measured from each fraction by gas chromatography. We evaluated the applicability of their *D*/*L* ratios for age estimation based on their correlation coefficient. A sex-related difference was observed in the *D*/*L* ratio. In particular, aged females showed a low ratio, suggesting an association with bone disorders. In males, the *D*/*L* ratios of acid-soluble peptide fraction showed the highest correlation rate ($r = 0.969$) with age, and those of total amino acid fraction showed the highest correlation rate ($r = 0.633$) with age in females. Without separation of male and female, the *D*/*L* ratios of total amino acid fraction showed the highest value ($r = 0.853$). The *D*/*L* ratio of acid-soluble peptide fractions differed according to the size of bone powder particles, being higher for larger particle sizes. These results suggest that the application of *D*/*L* ratio from total amino acid fraction is the most effective method for estimating age using the human femur. However, care is necessary when studing cadavers that might be females.

KEYWORDS: forensic science, forensic pathology, forensic anthropology, forensic medicine, age estimation, femur, racemization, aspartic acid, bone

Amino acids that constitute biological proteins are of the *L* form. However, conversion of the *L* to the *D* form increases with age in tissues showing slow metabolism such as teeth, lens, and parts of the brain (1–4). In particular, *D*-aspartic acid in dentin increases almost linearly with age (5–11), and is therefore used for age estimation from the teeth of unidentified cadavers (12). So far, the teeth are best for age estimation using racemization ratios (9); however, unidentified cadavers are sometimes recovered in a dismembered state. Then, age estimation should be done without teeth. In that case, bone is probably best for the age estimation because of its hardness. In fact, Ritz et al. (13,14) reported an increase of *D*-aspartic acid in cranial non-collagen proteins with age, and the applicability of this feature to the estimation of age. Pfeiffer et al. (15,16) separately examined acid-insoluble collagen fraction and acid-soluble peptides fraction from rib cartilage and cortical bone. However, we observed differences in the amounts of *D*-amino acid in acid-soluble peptide fractions isolated from teeth in accordance with the size of powdered tooth particles (unpublished data).

We evaluated the applicability of the *D*/*L* ratio from the femur for age estimation at death, separating male and female samples. In addition, differences of the *D*/*L* ratio in relation to the size of powdered bone particles was also examined.

Materials and Methods

Sampling and Preparation of Specimens

Compact femoral bone specimens that had been obtained from donated cadavers and fixed for less than one year in formalin were used. The middle area of the femur was cut using a cutter. The surface of the bone pieces was polished using a grindstone to remove flesh and spongy bone, washed, dried, and ground into powder using an agate mortar. Then, we sieved powdered bone particles with meshes to collect unisized particles. Powder samples with a particle size of 105 to 150 mμ obtained from 39 cadavers (21 males and 18 females aged 16 to 84 years) were used. Total amino acid fractions were measured in 10 mg of each powder sample placed in a test tube. For isolation of acid-insoluble collagen fraction and acid-soluble peptide fractions, 50 mg of the powder sample was mixed with 1 mL of 1 M hydrochloric acid and centrifuged at $4000 \times g$ at 5°C for 1 h. The sediment was collected as acid-insoluble collagen fraction, and the supernatant as acid-soluble peptide fraction. These fractions were dried using an evaporator.

For determination of differences in the *D*/*L* ratio according to the particle size, femoral compact bone obtained from a 49-year-old male (No. 8) was used. Four particle sizes (74–105, 105–150, 150–177, and 177–297 mμ) were examined. Total amino acid fractions were measured in 10 mg of powder with each particle size placed in a test tube. Acid-insoluble collagen fraction and acid-soluble peptide fractions were isolated using 100 mg of the sample by the same method as mentioned above.

Regression Model

The ratio of *D*- and *L*-aspartic acid was calculated from the areas under the eluted peaks.

$$D/L = (\text{area of } D\text{-aspartic acid})/(\text{area of } L\text{-aspartic acid})$$

The racemization of amino acids follows a first-order reversible rate law, where the racemization equation is:

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Received 11 June 1997; and in revised form 10 Sept. 1997, 17 Nov. 1997; accepted 30 Dec. 1997.

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

A linear regression model was constructed using this expression, where k is racemization rate constant, t is any given time during racemization, and the logarithmic term at $t = 0$ describes the amount of *D*-aspartic acid formed during hydrolysis. Regression lines deduced from the *D*/*L* ratios of total amino acid, acid-insoluble collagen, and acid-soluble peptide fractions, separately, but without separation of male and female. Then, estimated age was assessed according to the following formula:

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

where t is estimated age.

Results

Figure 1 shows a gas chromatogram of amino acids from femoral compact bone. *L*-aspartic acid was clearly separated from *D*-aspartic acid.

Table 1 and Fig. 2 show the relationship between the *D*/*L* ratio in total amino acid fractions, acid-insoluble collagen fraction, or acid-soluble peptide fractions and chronological age. The following racemization rate equation and its correlation coefficient (r) were obtained.

Total amino acid fraction:

Male, $\ln[(1 + D/L)/(1 - D/L)]_t = 0.000689t + 0.0336, r = 0.947, n = 21$

Female, $\ln[(1 + D/L)/(1 - D/L)]_t = 0.000304t + 0.0550, r = 0.633, n = 18$

Male and Female, $\ln[(1 + D/L)/(1 - D/L)]_t = 0.000559t + 0.0404, r = 0.853, n = 39$

Acid-insoluble collagen fraction:

Male, $\ln[(1 + D/L)/(1 - D/L)]_t = 0.000675t + 0.0291, r = 0.914, n = 21$

Female, $\ln[(1 + D/L)/(1 - D/L)]_t = 0.000185t + 0.0584, r = 0.418, n = 18$

Male and Female, $\ln[(1 + D/L)/(1 - D/L)]_t = 0.000518t + 0.0380, r = 0.790, n = 39$

Acid-soluble peptide fraction:

Male, $\ln[(1 + D/L)/(1 - D/L)]_t = 0.002918t + 0.0599, r = 0.969, n = 21$

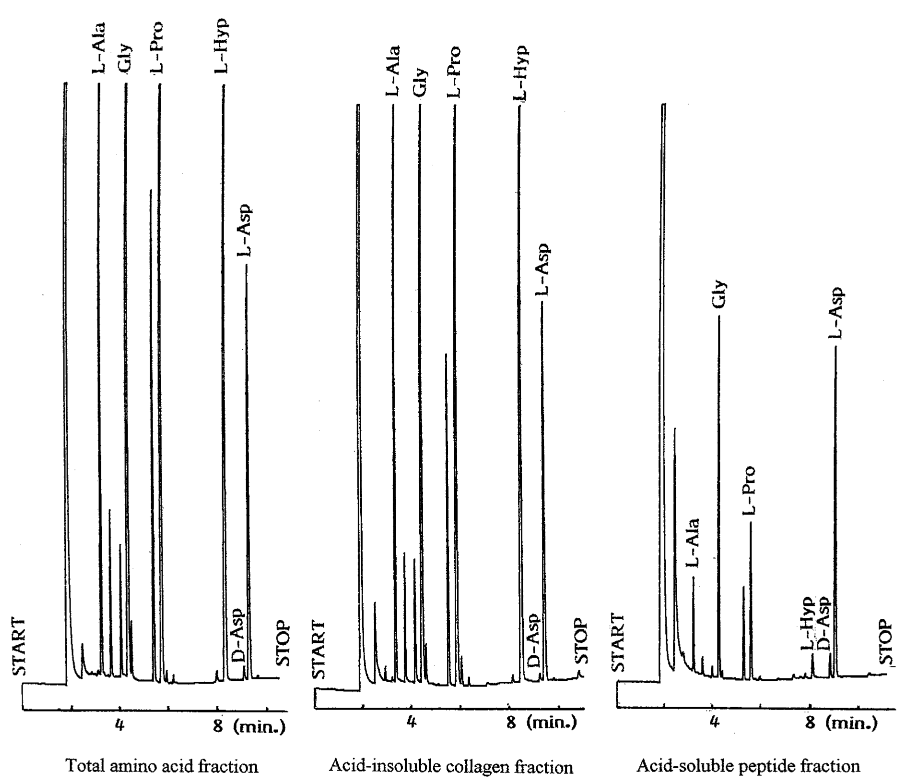

FIG. 1—*Gas chromatogram of amino acids of femoral bone.* L-*aspartic acid was clearly separated from* D-*aspartic acid after a retention time of about 9 min.*

TABLE 1—*Estimation of age from the human femur by determination of aspartic acid racemization.*

Individual No.	Sex	Actual age	TA Fraction D/L	TA Fraction Estimated Age	TA Fraction Error	IC Fraction D/L	IC Fraction Estimated Age	IC Fraction Error	SP Fraction D/L	SP Fraction Estimated Age	SP Fraction Error
1	M	16	0.0420	3	−13	0.0408	5	−11	0.0953	−5	−21
2	M	30	0.0500	17	−13	0.0482	20	−10	0.1620	29	−1
3	M	47	0.0612	37	−10	0.0540	31	−16	0.1922	44	−3
4	M	47	0.0722	57	+10	0.0624	47	±0	0.1989	48	+1
5	F	48	0.0674	48	±0	0.0618	46	−2	0.1826	39	−9
6	F	49	0.0680	49	±0	0.0672	56	+7	0.1906	43	−6
7	F	49	0.0686	50	+1	0.0666	55	+6	0.2068	52	+3
8	M	49	0.0688	51	+2	0.0632	49	±0	0.2268	62	+13
9	F	53	0.0772	66	+13	0.0744	70	+17	0.2424	70	+17
10	F	53	0.0714	55	+2	0.0644	51	−2	0.2341	65	+12
11	F	53	0.0694	54	−1	0.0676	57	+4	0.2170	57	+4
12	M	53	0.0706	54	+1	0.0612	45	−8	0.2098	53	±0
13	M	55	0.0742	60	+5	0.0684	59	+4	0.2104	53	−2
14	F	56	0.0824	75	+19	0.0808	83	+27	0.2324	64	+8
15	F	56	0.0720	57	+1	0.0680	58	+2	0.2485	73	+17
16	F	57	0.0664	47	−10	0.0650	52	−5	0.1848	40	−17
17	M	57	0.0782	64	+7	0.0670	56	−1	0.2290	63	+6
18	M	58	0.0756	63	+5	0.0748	71	+13	0.2412	69	+11
19	F	59	0.0738	60	+1	0.0656	53	−6	0.2326	65	+6
20	M	59	0.0782	68	+9	0.0714	64	+5	0.2290	63	+4
21	M	61	0.0746	61	±0	0.0670	56	−5	0.2243	60	−1
22	M	62	0.0778	67	+5	0.0762	74	+12	0.2507	74	+12
23	M	63	0.0758	63	±0	0.0700	62	−1	0.2249	61	−2
24	F	67	0.0706	54	−13	0.0698	61	−6	0.1926	44	−23
25	M	67	0.0776	67	±0	0.0662	54	−13	0.2558	76	+9
26	M	67	0.0828	76	+9	0.0810	83	+16	0.2460	71	+4
27	F	68	0.0764	64	−4	0.0748	71	+3	0.2226	60	−8
28	F	68	0.0734	59	−9	0.0732	68	±0	0.2349	66	−2
29	M	70	0.0830	76	+6	0.0744	70	±0	0.2704	84	+14
30	M	70	0.0800	68	+1	0.0764	74	+4	0.2658	81	+11
31	F	70	0.0742	60	−10	0.0734	68	−2	0.2104	53	−17
32	M	72	0.0871	84	+12	0.0854	92	+20	0.2505	74	+2
33	F	72	0.0756	63	−9	0.0696	61	−11	0.1757	36	−36
34	F	72	0.0816	74	+2	0.0680	58	−14	0.1930	45	−27
35	M	74	0.0794	70	−4	0.0734	68	−6	0.2847	91	+17
36	M	77	0.0800	71	−6	0.0750	71	−6	0.2890	93	+16
37	M	79	0.0865	82	+3	0.0858	92	+13	0.2998	98	+19
38	F	82	0.0853	80	−2	0.0776	76	−6	0.2584	78	−4
39	F	84	0.0766	65	−19	0.0690	60	−24	0.2187	58	−26
				$r = 0.853$			$r = 0.790$			$r = 0.702$	

NOTE—TA = total amino acid fraction; IC = acid-insoluble collagen fraction; SP = acid-soluble peptide fraction; $D/L = \ln[(1 + D/L)/(1 - D/L)]$; r = coefficient of correlation.

Female, $\ln[(1 + D/L)/(1 - D/L)]_t$

$$= 0.00276t + 0.1984,\ r = 0.125,\ n = 18$$

Male and Female, $\ln[(1 + D/L)/(1 - D/L)]_t$

$$= 0.001983t + 0.1045,\ r = 0.702,\ n = 39$$

where t is the actual age of the bone in years. The D/L ratio generally increased with age but was low in aged females. The correlation coefficients between the D/L ratio and age was highest in acid-insoluble peptide fractions from males and lowest in those from females. The values in total amino acid fractions appeared to be the most stable between male and female.

Figure 3 shows the D/L ratio according to particle size. The D/L ratios in total amino acid fractions and acid-insoluble collagen fraction did not differ among the particle sizes. However, the D/L ratio in the acid-soluble peptide fractions increased with particle size.

Discussion

Unidentified cadavers are sometimes recovered in a dismembered state. Ritz et al. (13,14) isolated the non-collagen protein fraction of the cranium and reported that *D*-aspartic acid in osteocalcin of purified bone (17,18) is closely related to the age at the time of death and allows, as with dentin, accurate estimation of age. However, their analysis method is complicated and requires a long time and special apparatus, and is therefore not appropriate for the practical estimation of age. In addition, when the cranium is recovered, and the teeth are present, age can be estimated from the dentin.

In studies of the racemization of amino acids, it is important to accurately separate *L*- from *D*-amino acids to obtain highly reproducible values. Figure 1 shows a gas chromatogram of amino acids from femoral compact bone. *L*-aspartic acid was clearly separated from *D*-aspartic acid. One characteristic of collagen is that it contains a large amount of hydroxyproline. The total amino acid fractions and acid-insoluble collagen fraction contained large amounts of hydroxyproline, reflecting the amino acid composition

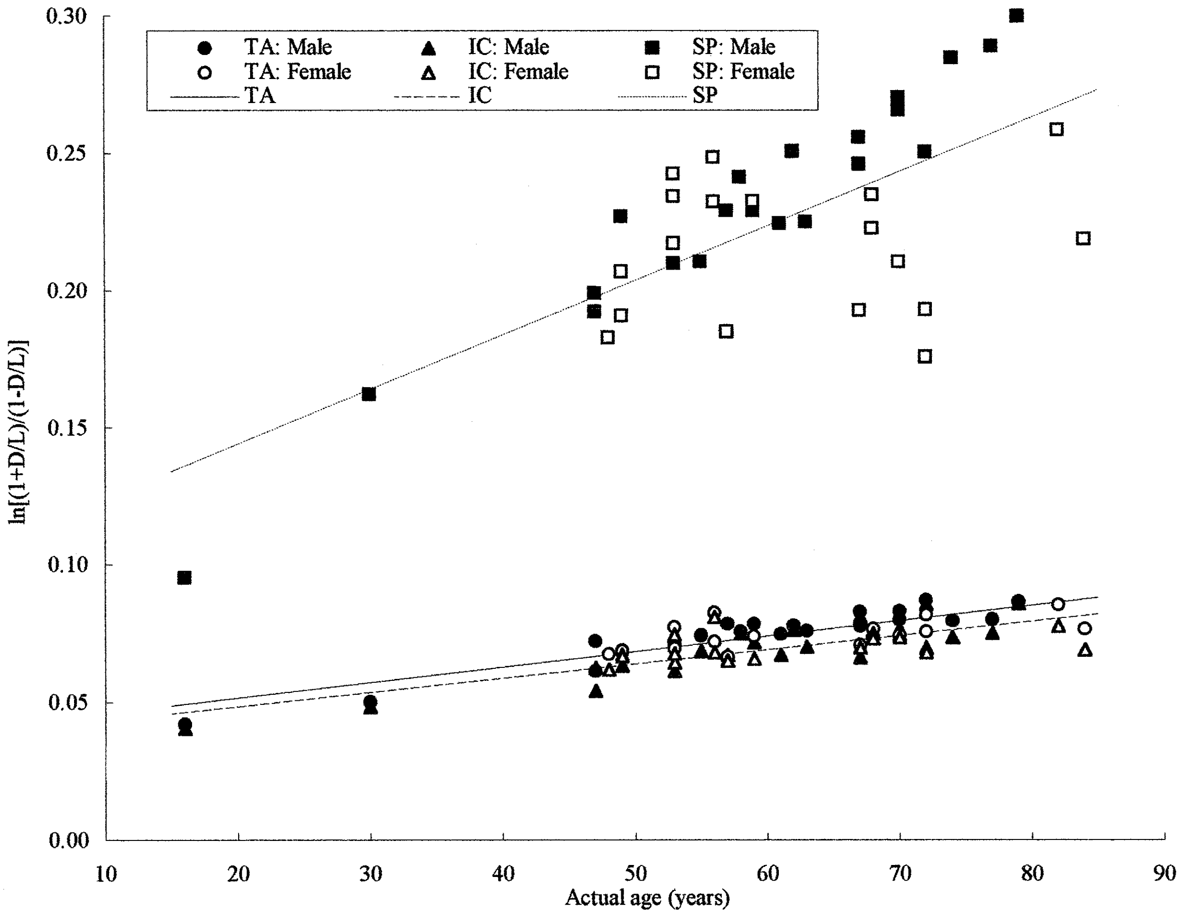

FIG. 2—*Correlation between the aspartic acid* D/L *ratio in femoral bone and actual age. TA = total amino acid fraction; IC = acid-insoluble collagen fraction; SP = acid-soluble peptide fraction. Lines are regression lines from each groups.*

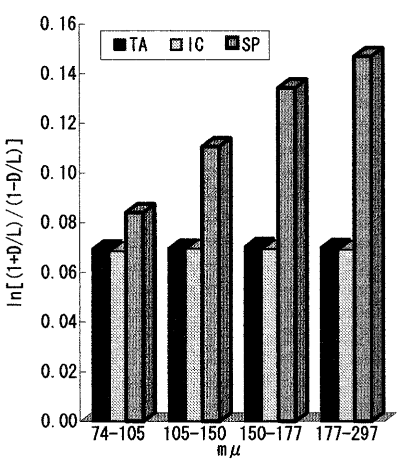

FIG. 3—D/L *ratios in total amino acid fraction, acid-insoluble collagen fraction, and acid-soluble peptide fraction according to bone powder particle size. The horizontal axis represents the powder particle size (mμ). TA = total amino acid fraction; CI = acid-insoluble collagen fraction; SP = acid-soluble peptide fraction.*

of collagen. However, the acid-soluble peptide fractions contained only a small amount of hydroxyproline.

The *D/L* ratios in the total amino acid fractions, acid-insoluble collagen fraction, and acid-soluble peptide fractions generally increased with age. However, they were reduced in aged females (Table 1, Fig. 2), probably due to bone disorders. In general, the incidence of metabolic bone diseases such as osteoporosis is high in aged females (19–21). The racemization reaction of amino acids advances gradually in proteins, rapidly in peptides, and most rapidly in free amino acids (22). This indicates that the *D/L* ratio in amino acid residues decreases after stronger washing of samples. In addition, since bone obtained from subjects with bone disorders is fragile, a larger amount of free amino acids with a high *D/L* ratio may be removed, resulting in a low *D/L* ratio. Concerning the low correlation between *D/L* ratio and age, Ritz et al. (13) suggested that the bone composition changes with age due to degradation. However, Pfeiffer et al. (15,16) showed a relatively high correlation even in the aged. Whether this discrepancy between the two studies is associated with the type of bone examined is not clear.

The *D/L* ratio did not differ according to the bone particle size in total amino acid fractions or acid-insoluble collagen fraction. However, the *D/L* ratio in the acid-soluble peptide fractions was higher in particles of larger size (Fig. 3). The sample of the No. 8 in Table 1 was the same as that used in Fig. 3. Therefore, the

D/*L* ratio should be the same. However, the *D*/*L* ratio in the acid-soluble peptide fractions was about 3.3 times higher than that in total amino acid fractions in the examination for estimation of age, and about 1.6 times higher that in the examination using different particle sizes. This is because acid-soluble peptide fractions were isolated using 50 mg samples in the former examination and using 100 mg samples in the latter. Thus, the *D*/*L* ratio in acid-soluble peptide fractions is very unstable and affected by the sample amount, concentration and amount of hydrochloric acid, and the manner of agitation after addition of hydrochloric acid to the sample. In addition, in the practical estimation of age using acid-soluble peptide fractions, accurate values may not be obtained unless the size of the powder particles is made uniform. Further studies of the reproducibility of this method using various types of bone are necessary.

For the estimation of age using femoral bone, the use of total amino acid fractions appears to be most effective in terms of ease of examination and reproducibility. Although this report is still preliminary, special care should be taken in applying this method to any remains that might be those of females, especially when the acid-soluble peptide fraction is applied.

Acknowledgments

We thank Dr. T. Yamamoto, the Department of Biology of our college for valuable suggestions.

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