TECHNICAL NOTE

Susumu Ohtani,[1] Ph.D.; Iwao Abe,[2] Ph.D.; and Toshiharu Yamamoto,[3] Ph.D.

An Application of D- and L-aspartic Acid Mixtures as Standard Specimens for the Chronological Age Estimation

ABSTRACT: Instead of using the control teeth, we tried to prepare standard specimens to derive the equations for calculation of the age (analytical curves) with respect to each kind of tooth of various ages. To prepare standard specimens, we determined the racemization ratio of the teeth of known age that had actually been used for the appraisal of chronological age (total control teeth). Then we mixed commercially available L-Asp and D-Asp in the same D/L ratio as the measured one in the total control teeth. As a result, we were able to obtain the racemization rate equations from the age-specific standard specimens of central and lateral incisors. These equations were closely similar to those derived from actual teeth of known age. Since the racemization rate equations obtained from the standard specimens were satisfactorily reproducible, we assumed that these equations could be used in place of those obtained from the control teeth. Actually, in the age estimation of unidentified corpses from teeth, the use of standard specimens enabled us to estimate the age almost as precisely as estimated using the control teeth. Thus, the present study has demonstrated that in the estimation of chronological age the control teeth can be substituted by the standard specimens. This shows the possibility of using the standard specimens also in other laboratories where the racemization ratio can be measured with sufficient reproducibility.

KEYWORDS: forensic science, age determination, dentin, racemization, D-aspartic acid, standard specimen

In 1976, Helfman & Bada (1) found a high correlation ($r = 0.979$) between the chronological age and the racemization ratio of aspartic acid (Asp) in the dentin of teeth. Subsequently this correlation was examined and confirmed by a number of researchers. It was reported that the racemization method using Asp in dentin was able to estimate the chronological age more accurately than conventional methods (2–17). The most serious drawback of the racemization method of the age estimation is that several control teeth (in this paper, control teeth mean natural teeth as controls) of the same kind as the specimen to be estimated are required (13). This situation is the same as that which occurs in electrophoresis for DNA tests requiring molecular weight markers. In the racemization method, D-Asp and L-Asp are separated and the ratio of their content is determined using gas chromatograph (GC), and the age is estimated on the basis of the ratio of D-Asp to L-Asp, i.e., the racemization ratio. Since only a minute amount of D-Asp is present, the D/L ratio must be determined to 4 decimal places. Accordingly subtle differences in heating temperatures between each experiment significantly affect the measured values, and it is difficult to consistently obtain stable values for the D/L ratio. For this reason, the control teeth were prepared in each case of appraisal

of age estimation. It imposes a fairly severe burden on us to keep healthy teeth of known age always on hand, however.

To solve this problem standard specimens (in this paper, standard specimens mean artificial mixtures of D-and L-Asp) were prepared using commercially available D-Asp and L-Asp, and their practicability was assessed.

Materials and Methods

Standard specimens were prepared on the basis of the racemization ratios in 70 central and 73 lateral incisors (known ages) from lower jaws, all of which had already been used for the estimation of chronological age as the control teeth (Tables 1 and 2 & Figs. 1–4) and had been extracted due to alveolar pyorrhea or other reasons. Their ages were all known, and caries had not extended to the dentin. One mm thick longitudinal sections were made by cutting the teeth around the middle in the labial-lingual direction (in the case of the incisors) with a low-speed cutter (Isomet, 11-1180, Buehler). Other areas except dentin were carefully removed out with the cutter and rinsed with ultrasonic waves sequentially in 0.2M HCl, distilled water (3 times), ethanol and ethyl ether for 5 min respectively. Then the dentin sections were pulverized in an agate mortar (*ca.* 20 mg per tooth), and 10 mg of the powder was used as the specimen for determination of the racemization ratio (whole dentin).

The content of D-Asp was determined on the basis of the content of L-Asp and the racemization ratio: $\ln[(1 + D/L)/(1 - D/L)]$. By the conventional method (7) the specimen was analyzed with a GC (GC-17A, Shimadzu, Kyoto) after hydrolysis and derivatization.

TABLE 1—*Preparation of standard specimens for the central incisor.*

t=	A	B (mg)	C	Error (A-C)	Probability	D	E (mg)	F	Error (A-F)	Probability
20	0.06252	62.52	0.0671	−0.0046		0.05902	59.02	0.0632	−0.0007	
30	0.07468	74.68	0.0772	−0.0025		0.07118	71.18	0.0744	0.0003	
40	0.08684	86.84	0.0898	−0.0030	$t_{(5)} = 7.12$	0.08334	83.34	0.0871	−0.0003	$t_{(5)} = 0.20$
50	0.09900	99.00	0.1005	−0.0015	P: 0.01−0.001	0.09550	95.50	0.0998	−0.0008	P: 0.8−0.9
60	0.11116	111.16	0.1144	−0.0032		0.10766	107.66	0.1098	0.0014	
70	0.12332	123.32	0.1271	−0.0038		0.11982	119.82	0.1236	−0.0003	

A: Racemization ratio obtained by substituting t in the equation $\ln[(1 + D/L)/(1 − D/L)]_t = 0.001216t + 0.0382$ with each assumptive age (t).

B: Content of D-Asp in 1000 mg of L-Asp before correction.

C: Racemization ratio obtained from the hydrolysates of the standard specimens. The racemization rate equation is expressed as $\ln[(1 + D/L)/(1 − D/L)]_t = 0.001207t + 0.0417$.

D: Corrected value obtained by subtraction of 0.0035 from the racemization ratio of each assumptive age (A).

E: Corrected value of D-Asp content in 1000 mg of L-Asp.

F: Racemization ratio obtained from the hydrolysates of the standard specimens prepared using the corrected values.

TABLE 2—*Preparation of standard specimens for the lateral incisor.*

t=	A	B (mg)	C	Error (A-C)	Probability	D	E (mg)	F	Error (A-F)	Probability
20	0.06256	62.56	0.0665	−0.0039		0.05916	59.16	0.0621	0.0005	
30	0.07484	74.84	0.0780	−0.0032		0.07144	71.44	0.0740	0.0008	
40	0.08712	87.12	0.0899	−0.0028	$t_{(5)} = 9.23$	0.08372	83.72	0.0874	−0.0003	$t_{(5)} = 2.54$
50	0.09940	99.40	0.1010	−0.0016	P < 0.001	0.09600	96.00	0.0978	0.0016	P:0.1−0.05
60	0.11168	111.68	0.1147	−0.0030		0.10828	108.28	0.1112	0.0005	
70	0.12396	123.96	0.1276	−0.0036		0.12056	120.56	0.1233	0.0007	

A: Racemization ratio obtained by substituting t in the equation $\ln[(1 + D/L)/(1 − D/L)]_t = 0.001216t + 0.0382$ with each assumptive age (t).

B: Content of D-Asp in 1000 mg of L-Asp before correction.

C: Racemization ratio derived from the hydrolysates of the standard specimens. The racemization rate equation is expressed as $\ln[(1 + D/L)/(1 − D/L)]_t = 0.001219t + 0.0414$.

D: Corrected value obtained by subtraction of 0.0034 from the racemization ratio of each assumptive age (A).

E: Corrected value of D-Asp content in 1000 mg of L-Asp.

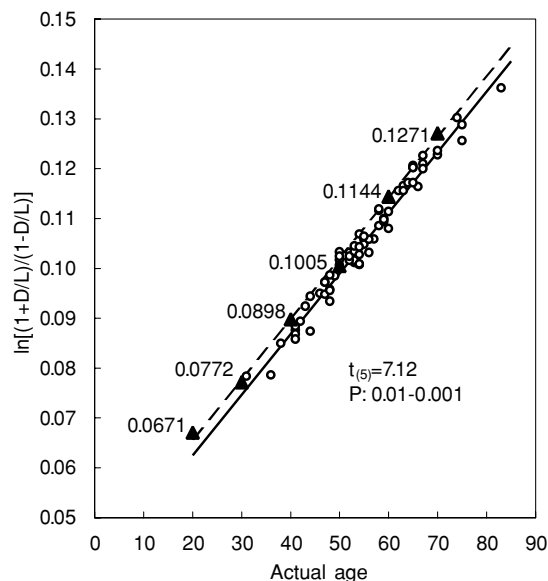F: Racemization ratio obtained from the hydrolysates of the standard specimens prepared using the corrected values.

FIG. 1—*Comparison between analytical curves derived from the central incisors (○ & solid line) and those derived from the hydrolysates of the standard specimens before correction (▲ & broken line).*
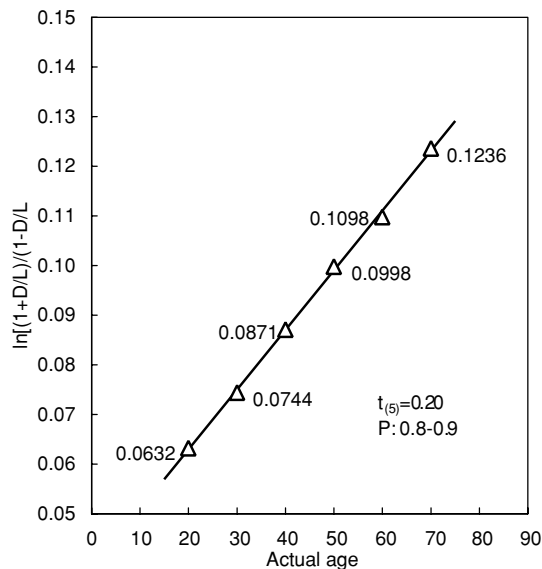

FIG. 2—*Comparison between analytical curves derived from the central incisor (solid line) and those derived from the hydrolysates of the standard specimens after correction of D/L ratio (△). Those two lines become almost the same line.*

The column of the GC was 30 m in length, consisting of a capillary column (internal diameter: 0.3 mm) lined with Chirasil-Val (18).

Plotting the chronological age on X axis, the racemization ratio on Y axis, we derived the following linear regression equation by the least-square method:

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

in which $\ln[(1 + D/L)/(1 − D/L)]$ represents the log-transformed racemization ratio, t the chronological age, and k the racemization rate constant. To estimate the chronological age, we plotted the age on the Y axis and the racemization ratio on the X axis, and derived the following linear regression equation by the least square method:

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

FIG. 3—*Comparison between analytical curves derived from the lateral incisors (○ & solid line) and those derived from the hydrolysates of the standard specimens before correction (▲ & broken line).*

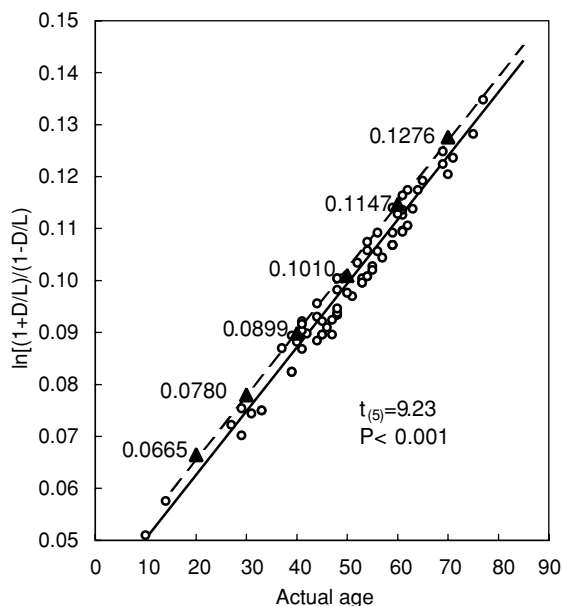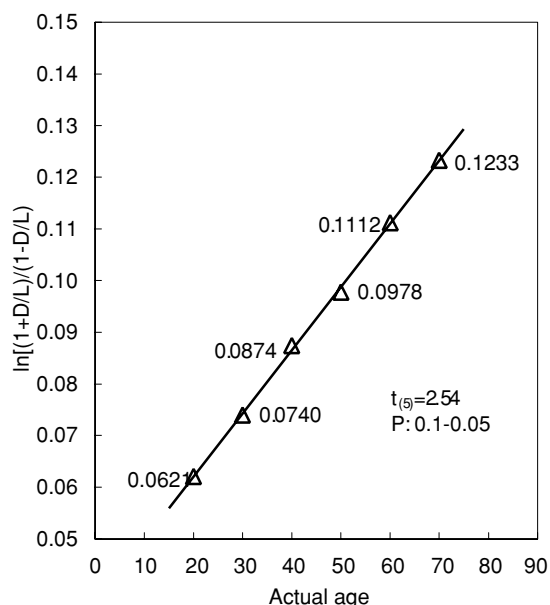

FIG. 4—*Comparison between analytical curves derived from the lateral incisor (solid line) and those derived from the hydrolysates of the standard specimens after correction of D/L ratio (△ & broken line). Those two lines become almost the same line.*

The estimated age was obtained by substituting the D/L ratio in this linear regression equation with that of the specimen to be estimated.

Preparation of Standard Specimens

As for the central incisor (Table 1), the following racemization rate equation was derived on the basis of the racemization ratio of 70 central incisors (Fig. 1):

$$\ln[(1 + D/L)/(1 - D/L)]_t = 0.001216t + 0.0382 \qquad (1)$$

In the case of 20 years of an assumptive age, substituting t in this equation by 20 yields 0.06252 as the value of $\ln[(1 + D/L)/(1 - D/L)]_t$ (Table 1-A). On the basis of this result, a standard specimen was prepared by mixing commercially available L-Asp (WAKO K.K., Osaka) with D-Asp (WAKO, K.K.) in the ratio of 1000 mg: 62.52 mg (Table 1B) for an example. The mixture was dissolved in 50 mL of 0.5 M HCl and kept refrigerated. When it was used 10 μL f the standard solution dissolved in HCl was put into a 10 mL test tube equipped with a screw, hydrolyzed by the conventional method (7), N-TFA isopropyl esterificated, and analyzed on a gas chromatograph. The same analysis was performed with respect to the specimens of other assumptive ages (Table 1-t). By hydrolysis and analysis with the gas chromatograph of the standard solution of a mixture of L-Asp and D-Asp, the D/L ratios shown in Table 1-C were obtained. From these values, the following racemization rate equation was derived (Fig. 1):

$$\ln[(1 + D/L)/(1 - D/L)]_t = 0.001207t + 0.0417 \qquad (2)$$

Statistical analysis (19) of the difference in the racemization ratio between the control teeth and the standard specimens (Table 1, A–C) yielded $t_{(5)} = 7.12$ (P: 0.01–0.001), showing a significant difference. The difference could be ascribed to racemization during hydrolysis. Comparing equations (1) and (2), reaction rate constants were almost same (0.001216 vs. 0.001207), however, constants were considerably different (0.0382 vs. 0.0417). Therefore, we concluded that it was required to correct the constants to adjust the racemization rate equation derived from standard samples to the equation derived from control teeth. Accordingly, to correct the difference we subtracted the value of $\ln[(1 + D/L)/(1 - D/L)]_{t=0}$ (0.0382) in the rate equation of the control teeth from that of $\ln[(1 + D/L)/(1 - D/L)]_{t=0}$ (0.0417) in the rate equation of the standard specimen, and obtained 0.0035. Subtraction of 0.0035 from each value in Table 1-A yielded the values in Table 1-D. On the basis of these values, the corrected contents of D-Asp in 1000 mg of L-Asp are shown in Table 1-E. Again we prepared standard specimens using the corrected values shown in Table 1-E. These specimens were reanalyzed with the chromatograph after hydrolyzation, yielding the D/L ratios shown in Table 1-F. The racemization rate equation fitting with these new standard specimens was derived as:

$$\ln[(1 + D/L)/(1 - D/L)]_t = 0.001203t + 0.0389 \qquad (3)$$

This rate equation was not significantly different from that derived from the control central incisors ($t_{(5)} = 0.2$, P: 0.8–0.9, Table 1 & Fig. 2).

Application of the same procedure to the lateral incisors was performed. The racemization rate equation derived from 70 natural lateral incisors was follows (Table 2 & Fig. 3):

$$\ln[(1 + D/L)/(1 - D/L)]_t = 0.001219t + 0.0414 \qquad (5)$$

Again, the reaction rate constants were almost same, but the constants were different (0.0380 vs. 0.0414). Subtraction of 0.038 from 0.0414 yielded 0.0034. Correction was performed by the subtraction of 0.0034 from each racemization rate of assumptive ages (Table 2-D).

The racemization rate equation derived from the corrected standard specimens was as follows:

$$\ln[(1 + D/L)/(1 - D/L)]_t = 0.001223t + 0.0376 \qquad (6)$$

As a result, rate equations of (4) and (6) were not significantly different (Table 2 & Fig. 4).

TABLE 3—*Examples of age estimation using the standard specimens instead of the control teeth.*

		Case #1 (37 years of age)			Case #2 (49 years of age)		
		E	F	G	E	F	G
		20	0.0622	19	20	0.0620	20
		30	0.0748	30	30	0.0746	30
		40	0.0865	39	40	0.0859	39
A							
		50	0.0987	49	50	0.0978	49
		60	0.1111	59	60	0.1102	59
		70	0.1235	70	70	0.1233	70
		1[*1]	0.0838	37	2[*3]	0.095	47
B							
		1[*2]	0.0879	40			
C		$t = 818.45R - 31.00$			$t = 822.76R - 30.94$		
		R = 0.999			r = 0.999		
D		38 years of age			47 years of age		

A: Standard specimens; B: Natural teeth for age estimation; C: Age estimation equation derived from standard specimens; D: Results of age estimation; E: Standard specimens & natural teeth for age estimation; F: Racemization ratio; G: Age estimated from analytical curve (C) derived from the standard specimens; t, estimated age; R: $\ln[(1 + D/L)/(1 - D/L)]$; [*1]:Right mandibular central incisor; [*2]: Left mandibular central incisor; [*3]: Right mandibular lateral incisor.

Results and Discussion

The standard specimens were assessed with respect to the central and lateral incisors, because they were single-rooted, their whole dentin could easily be collected due to their small size, and the correlation between their racemization ratio and the chronological age was high (5). The racemization reaction is strongly affected by temperature (20). Heating steps in processing of the specimen include hydrolysis (100°C for 6 h), drying in a rotary evaporator (40~45°C for 20 min, twice), derivatization (100°C for 35 min) and analysis with a gas chromatograph (90~140°C for *ca.* 12 min). In particular, 1°C difference in the hydrolysis temperature results in such a difference as *ca.* 1.5 years in the estimated age (21). Accordingly, the processing of the standard specimens from hydrolysis to chromatographic analysis was performed at the same temperature as that of the specimen for age estimation. The racemization ratios of Asp of the age-specific standard specimens of the central and lateral incisors increased linearly with age, and almost the same racemization rate equations were obtained as those derived from actual teeth. The equations showed a satisfactory reproducibility, suggesting that the standard specimens could be used in place of the control teeth.

Table 3 shows the results of actual age estimation from the teeth of unidentified corpses using the standard specimens instead of the control teeth. Case #1 was estimated to be 38 years of age, who subsequently proved to be 37 years of age, Case #2 was estimated to be 47 years of age, and later turned out to be 49 years of age. Thus, by using the standard specimens it was possible to estimate the age as accurately as the conventional age estimation method using the control teeth. The use of the standard specimens has the advantages that not only are they available whenever an appraisal is necessary but also that they can always be obtained with respect to many standard ages (n = 6 in the present study). Thus, the analytical curves would become more reliable.

Not only intralaboratory, but also interlaboratory standardization, amino acid standards, dentin pool samples and control teeth are proposed to be required (22). In the present study, we assessed the application of amino acid standards for the chronological age estimation considering the procedure steps that probably have principal effects on D/L ratio. In fact, the mixing ratios of D-Asp and L-Asp for the standard samples calculated on the basis of our data were required to correct for the application. Thus, these values would be different among research workers and laboratories, because a minute difference in the temperature and period of hydrolysis would yield different values of D/L ratio. However, the present study indicated the possibility of the application of amino acid standards instead of control teeth for the chronological age estimation using racemization rates.

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Additional information and reprint requests:
Susumu Ohtani, Ph.D.
Department of Forensic Medicine
Kanagawa Dental College
82 Inaoka-cho, Yokosuka, Kanagawa 238-8580
Japan
Fax: +81-46-822-8801
E-mail: ohtanisu@kdcnet.ac.jp