Original article

Age estimation using aspartic acid racemization in various forensic samples: a preliminary study

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Objectives To evaluate the potential of aspartic racemization to estimate age using different types of forensic specimens.

Methods Samples of blood, vitreous fluid and teeth were collected from cadavers and placed in 6 M hydrochloric acid before o-phthaldialdehyde- N-acetyl-L-cysteine (OPA-NAC) derivatization. D- and L-aspartic acid were determined by high performance liquid chromatography using a fluorescence detector. Linear regression was used to evaluate the relationship between the ln [(1+D/L)/(1-D/L)] value and chronological age.

Results A higher correlation was observed between aspartic acid racemization rates and age in dentin than in either vitreous fluid or blood with calculated error ranges of ± 4.5, 22.6 and 581.4 years, respectively. Dentin had a lower amino acid turnover rate than either the vitreous fluid or blood samples.

Conclusions Dentines can provide a more accurate estimation of age than either vitreous fluid or blood. Other low amino acid turnover rate tissues should be studied to determine their age estimation precision capacity. **Chiang Mai Medical Journal 2020;59(2):53-9.**

Keywords: age estimation, aspartic acid racemization, d-aspartic acid, l-aspartic acid, forensic samples

Introduction

Identification of unknown cadavers is an essential part of postmortem examination and can include determination of broad categories, e.g., sex, height, race, and age. Age estimation is frequently used in crime investigations and following mass disasters in order to obtain correct identities. Methods to determine biological age in adults have been difficult and results have often been inconclusive (1).

Previous studies have focused on biochemical alterations to determine the age of adults. Helfman and Bada reported that aspartic acid in human tooth enamel can be used to evaluate age based on racemization (r = 0.921) by measuring D-aspartic acid accumulation (2). Additionally, age estimation based on amino acid racemization of Dentine, enamel or a whole tooth have been demonstrated to provide D/L ratios that are highly correlated with actual age (r = 0.93-0.98)(3,4).

Aspartic acid racemization in human teeth is one of the most reliable methods for determining chronological age. However, sample collection takes a long time. Also, dental caries present a problem for analysis because it can potentially affect the rate of protein degradation and thus introduce a risk of misinterpretation (5). In cases where unidentified bodies are found to have extensive dental caries or to be toothless, age estimation based on teeth is not possible.

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Change in amino acid racemization in other human tissues which have a protein structure (6), which were formed early in life and which have a low amino acid turnover rate can also be used. For example, studies have found that in Dentine, cementum, human lenses, brain white matter, and intervertebral discs there is a high correlation between aspartic acid racemization and age (7). The concentration of other biochemicals in vitreous fluid is also related to the postmortem interval (8), so blood and vitreous fluids are potentially also suitable for postmortem investigation of D/L aspartic acid ratios. Obtaining these types of specimens is easier than teeth, but few studies of their efficacy have been reported.

Objectives

The aim of this study was to assess aspartic racemization in different types of specimens including blood, vitreous fluid and teeth samples from cadavers. The appropriate samples could be used and to evaluate their efficacy in forensic age estimation.

Methods

Chemicals

D- and L-aspartic acid, o-phthaldialdehyde and N-acetyl-L-cysteine were all purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Boric acid, sodium tetraborate, sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from RCI Labscan (Bangkok, Thailand).

Reagent preparation

The D- and L-aspartic acid were dissolved in borate buffer pH 9. O-phthaldialdehyde (OPA)/ N-acetyl-L-cysteine (NAC) reagent was prepared by dissolving OPA (5.5 mg) in methanol, then adding 0.4 M sodium borate buffer pH 9 (500 µL) and 1 M NAC solution (120 µL).

Instrumentation and operating procedures

The high-performance liquid chromatography system used (HPLC; Agilent 1260 infinity) consisted of a G1312B binary pump, a G4225A degasser, a G1367E autosampler, a G1316C thermostat compartment, and a G1321B fluorescence detector. D- and L-aspartic acid were measured at an excitation wavelength of 337 nm and an emission wavelength of 442 nm using a fluorescence detector following Monum et al. (9). The derivatizations of the amino acid isomer were separated using a Merck Purospher® C8 column (150 x 4.60 mm, 5 µm). A gradient elution was carried out with 30 mM sodium phosphate buffer pH 5.5 and also with methanol: 0-3 min (85%A), 3-10 min (85-15 %A) and 10-15 min (15-85%A). The flow rate of the mobile phase was 0.7 mL/min and the injection volume was 10 µL.

Sample preparation and derivatization

Samples of Dentine (n=10), vitreous fluid (n=9) and blood (n=10) were collected from cadavers at the Department of Forensic Medicine; the Research Committee of the Faculty of Medicine, Chiang Mai University approved this study. The subjects ranged in age from 14-70; decomposed or skeletonized bodies were excluded. The Dentine samples were cut using a low-speed cross section saw, then the enamel layer was separated using a dental diamond disc and a burr under cool temperatures. The Dentine samples were broken into small pieces then crushed into powder before the hydrolysis step. The Dentine powder, the vitreous fluid and the blood were all hydrolysed for 20 hours with 6 M hydrochloric acid. The samples were then evaporated and diluted with 0.3 M sodium phosphate buffer pH 7.5. Derivatization was carried out by mixing 100 µL of the sample solution with 200 µL of the OPA-NAC derivatization reagent. After five minutes, 200 µL of sodium phosphate buffer was added and the solution was allowed to stand at room temperature for five minutes before HPLC analysis.

Data analysis

Estimated age was calculated using the equation $\ln[(1+D/L)/(1-D/L)] = (m \times \text{age}) + b$ where m is the rate constant of racemization and b is the constant value. Values of m and b were obtained from the linear regression between ln[(1+D/L)/(1-

D/L)] and age. Comparison of D/L, ln[(1+D/L)/(1-D/L)] and the error between groups was conducted using one-way ANOVA following a post-hoc test.

Results

Table 1 shows the results of the actual age, D/L ratio, calculated D/L value, calculated age and error in each type of sample. The peaks of the D-and L-aspartic acid were clearly separated from the HPLC chromatogram in each sample and shown in Figure 1. The peak of the D- and L-aspartic acid was clearly separated from vitreous fluid, blood and Dentine samples in reversed-phase chromatography . The retention times for D- and L-aspartic acid were 8.18 and 8.91 min, respectively.

Linear regression analysis showed a significant correlation between the Dentine and ln[(1+D/L)/(1-D/L)] values and actual age ($p < 0.0001$). The

Table 1. Experimental data from vitreous fluid, blood and dentine samples

Sample	Actual age (years)	D/L	ln[(1+D/L)/(1-D/L)] (years)	Calculated age (years)	Error
Vitreous fluid					
1	44	0.6000	1.3863	68	24
2	33	0.4286	0.9163	41	8
3	58	0.3200	0.6633	27	30
4	70	0.6929	1.7069	86	16
5	14	1.0000	ND	ND	ND
6	30	0.1282	0.2578	4	26
7	38	0.6533	1.5622	78	40
8	65	0.4714	1.0238	47	17
9	25	0.1560	0.3146	8	17
Blood					
1	54	0.0211	0.0422	594	540
2	78	0.2080	0.4222	119	41
3	16	0.2149	0.4366	101	85
4	37	0.4844	1.0574	675	638
5	14	0.4086	0.8679	438	424
6	56	0.0101	0.0201	621	565
7	23	0.0857	0.1718	432	409
8	62	0.8182	2.3026	232	2,170
9	30	0.0929	0.1863	414	384
10	72	0.0064	0.0129	630	558
Dentine					
1	50	0.0258	0.0517	47	3
2	30	0.0296	0.0459	35	5
3	55	0.0283	0.0565	57	2
4	88	0.0349	0.0698	83	5
5	53	0.0293	0.0586	61	8
6	50	0.0265	0.0530	50	0
7	23	0.0173	0.0346	13	10
8	37	0.0247	0.0495	43	6
9	36	0.0235	0.0470	38	2
10	29	0.0204	0.0407	25	4

ND; not detected

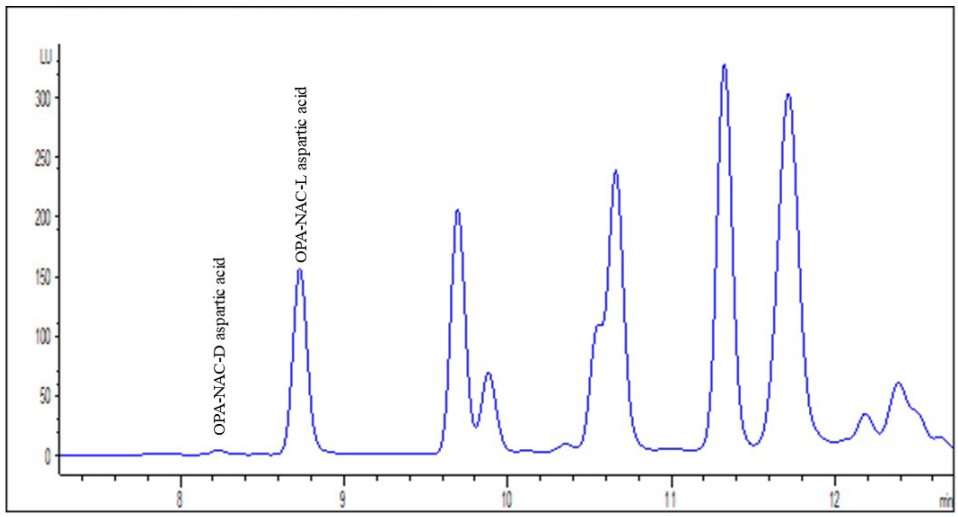

Figure 1. HPLC chromatogram of OPA-NAC D- and L-aspartic acid. X axis indicates time (minutes) and Y axis demonstrates intensity (Luminescence)

correlation coefficient (r) was 0.959 and the coefficient of determination (r2) was 0.920. The ln [(1+D/L)/(1-D/L)] value for vitreous fluid was not significantly correlated with chronological age ($p = 0.164$). The correlation coefficient (r) was 0.544 and the coefficient of determination (r2) was 0.295.

As illustrated in Table 2, the mean values of D/L, ln [(1+D/L)/(1-D/L)] and the error value in Dentine were significantly different from those in vitreous fluid and blood. The D/L and error value in vitreous fluid also differed from blood, indicating Dentine might be a more suitable material for chronological age estimation.

Discussion

Age estimation in living persons, cadavers and skeletal remains is one of the main problems in forensic science (10). Morphological and histological methods are the major techniques for age estimation (11). Amino acid racemization is the conversion of pure L- amino acid form into the racemic mixture of D- and L- forms; this is a reversible reaction and acts as a first-order kinetic reaction (12, 13). D/L, an amino acid enantiomeric ratio, gives the estimated age of protein (14). The use of aspartic acid, a non-essential amino acid, in racemization is a chemical method for age estimation which is currently one of the most

Table 2. Descriptive statistics of experimental data from vitreous fluid, blood and dentine samples

Values	Vitreous fluid	Blood	Dentine
D/L			
Mean ±SD	0.49±0.28*	0.24±0.26	0.02±0.01*,**
95% CI	0.281-0.708	0.047-0.424	0.022-0.029
ln[(1+D/L)/(1-D/L)]			
Mean ±SD	0.98±0.55	0.55±0.71	0.05±0.01*,**
95% CI	0.521-1.437	0.042- 1.061	0.437-0.578
Error value			
Mean ± SD	22.65±9.81*	581.40±592.67	4.5±2.99*,**
95% CI	14.450-30.850	157.431-1005.369	2.361-6.6394

*Statistically significant when compared with blood

**Statistically significant when compared with vitreous fluid

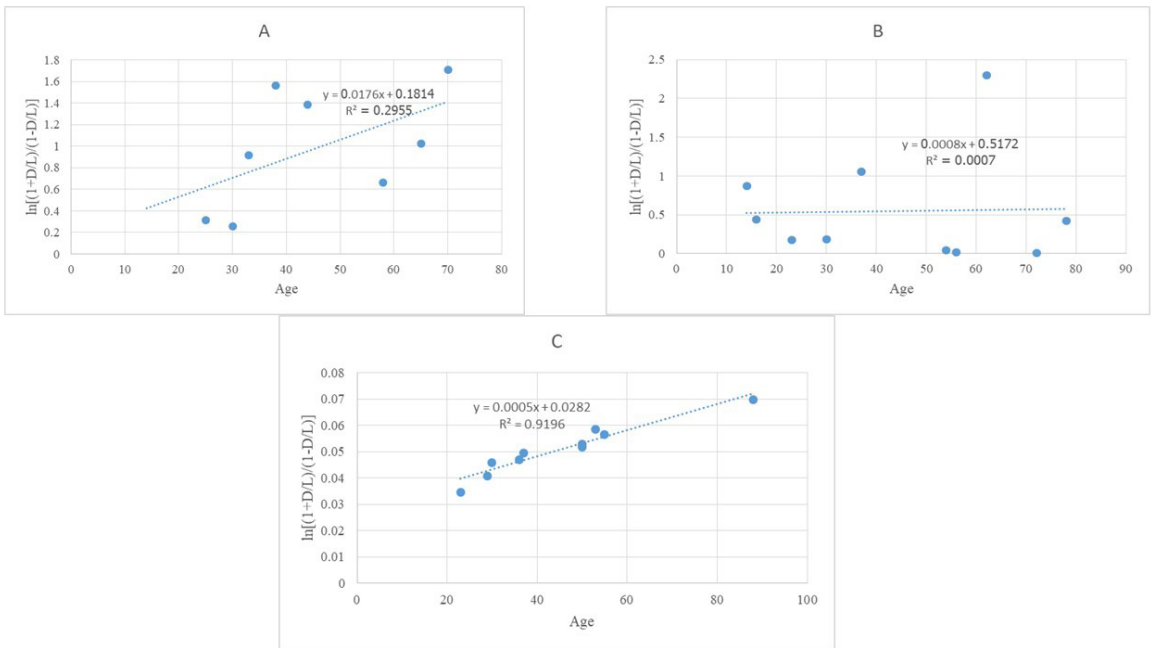

Figure 2. Plot of ln[(1+D/L)/(1-D/L)] of aspartic acid against the age of samples in the linear regression line. (A) vitreous fluid, (B) blood, (C) dentin

accurate methods available (15). Aspartic acid racemization can be used to estimate the age of fresh cadavers as well as skeletal remains. Over the past decade, HPLC has been a commonly used instrument for D- and L-aspartic acid isomer quantification. Derivatization of amino acid isomers using the OPA-NAC method is an easy procedure with high sensitivity in age estimation models (16). Temperature, pH, ionic strength, metal ion chelation and heating times are all important factors which can affect the amino racemization rate (17).

In this study, nine samples of vitreous fluid and ten samples of blood and Dentine from bodies obtained during post-mortem examinations in the Department of Forensic Medicine were used to investigate the correlation between the aspartic |acid racemization ratio and chronological age. The vitreous fluid and blood samples were easier to collect and prepare for the age estimation process than Dentine; however, results showed that the ln[(1+D/L)/(1-D/L)] values of aspartic acid in Dentine were more closely related to the chronological age.

The rate of aspartic acid racemization varies with the type of protein structure. In this study, the difference between the calculated age and the actual age was 16-17 years in vitreous fluid, 41-2,170 years in blood and 0-10 years in Dentine. Dentine gave significantly more accurate age estimates than either vitreous fluid or blood specimens. Dentine in teeth is a well-preserved hard substance that changes very slow (18), whereas, vitreous fluid liquifies relatively rapid with aging (19) as a result of the destruction of collagen fibrils which might possibly affect the D/L aspartic racemization. Also, erythrocytes are at various stages of maturation, so their composition is not uniform (20). In our study, Dentine was found to be the most accurate for estimating age based on aspartic acid racemization because of the very low racemization rate in Dentine resulting from the lack of change in protein structure. Additionally, food and/or protein consumption can affect the racemization rate of L-formed aspartic acid in the bloodstream (21). In this study, the D/L ratio of Dentine was much lower than that of both vitreous fluid and blood which suggests that the turnover rate

of D-formed to L-formed aspartic acid in Dentine is very low (ratio = 0.02), while vitreous fluid and blood have a high turnover rate (0.49 and 0.24, respectively). Other tissues with low amino acid turnover rates should be studied to determine their age estimation capability.

A limitation of this study is the small sample size. A larger sample might give a different correlation between D/L ratio and age.

Conclusions

The D/L ratio of the aspartic acid racemization of Dentine is more highly correlated with chronological age than either vitreous fluid or blood.

Acknowledgments

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Conflict of interest

The authors report no potential conflicts of interest.

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การประมาณอายุด้วยวิธีเรซิไมเซชันของกรดแอสพาร์ติกจากสิ่งส่งตรวจทางนิติเวชชนิดต่าง ๆ : การศึกษาเบื้องต้น

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วัตถุประสงค์ เพื่อประเมินอายุด้วยใช้วิธีเรซิไมเซชันกรดเอสพาร์ติกจากสิ่งส่งตรวจทางนิติเวชชนิดต่าง ๆ

วิธีการ เก็บตัวอย่าง เลือด น้ำวุ้นตา และฟันจากผู้เสียชีวิต จากนั้นตัวอย่างถูกไฮโดรไลซิสด้วยกรดไฮโดรคลอริกเข้มข้น 6 โมลาร์แล้วนำมาสร้างอนุพันธ์ด้วย ออโธ-ฟาธิลไดอัลดีไฮด์และอะซิทิลซิสเทอีน ปริมาณ ดี และแอล กรดแอสพาร์ติก ถูกนำมาวัดโดยเครื่องโครมาโทกราฟีสมรรถภาพสูงและใช้ฟลูออเรสเซ็นต์เป็นตัวตรวจวัด ข้อมูลที่ได้นำมาวิเคราะห์ทางสถิติและสร้างสมการถดถอยเชิงเส้นระหว่างลอการิทึมธรรมชาติของค่า [(1+ดี/แอล กรดแอสพาติก)/(1-ดี/แอล กรดแอสพาติก] กับอายุของตัวอย่าง

ผลการศึกษา ความสัมพันธ์ระหว่างอายุและอัตราส่วนของเรซิไมเซชันกรดแอสพาร์ติกในเนื้อฟันมีค่าสูงกว่าน้ำวุ้นตาและเลือด ซึ่งมีค่าความคลาดเคลื่อนเท่ากับ ± 4.5, 22.6 และ 581.4 ปี ตามลำดับ เนื่องจากเนื้อฟันนั้นมีอัตราการพลิกกลับกรดอะมิโนต่ำกว่าน้ำวุ้นตาและเลือด

สรุป เนื้อฟันเป็นตัวอย่างที่เหมาะสมสำหรับการประมาณอายุมากกว่าน้ำลูกตาและเลือด เนื้อเยื่อที่มีอัตราการพลิกกลับของกรดอะมิโนต่ำอื่น ๆ ควรมีการศึกษาเพิ่มเติมเพื่อประมาณอายุโดยวิธีแอสพาร์ติกแอซิดเรซิไมเซชัน **เชียงใหม่เวชสาร 2563; 59(2):53-9.**

คำสำคัญ: การประมาณอายุ กรดแอสพาร์ติกเรซิไมเซชัน ดี-กรดแอสพาร์ติก แอล-กรดแอสพาร์ติก สิ่งส่งตรวจทางนิติเวช