Evaluated Age Estimation Using Aspartic Amino Acid Racemization from Skull and Sternum for Forensic Application

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ABSTRACT

Objective: A precisely estimated age will reduce the time and cost of identifying unknown deceased persons. At present aspartic acid racemization (AAR) of dentin is the best method for age estimation from body remains. However, skeletal or part of body remains may not be available for analysis by the Dextro/Levo (D/L) ratio. Thus, age estimation by using AAR from other organs, for example, bones, is necessary. The present study evaluated age estimation methods using aspartic amino acid racemization from the skull and sternum in the Thai population.

Design: To examine the total amino acid of skull and sternum by using the AAR technique.

Materials and Methods: Bone samples were collected from autopsy cases. Forty pieces of temporal bone and manubro-sternum from each region were used to analyze the D/L ratio.

Results: The age estimation equation of the skull age was ((K- 0.0203)/0.0005)), which R² and R were 0.511, 0.7151 respectively and SEE was 17.46 years, while the age estimation equation of the sternum was ((K- 0.0095)/0.0003)), R² was 0.277 and R was 0.527 and SEE was 31.52 years.

Conclusions: The skull was not a good specimen for age estimation by using the AAR method. However, this method may be beneficial in the case of fragmented skull remains. The sternum may not be suitable for use in age estimation by using the AAR technique in a forensic context.

KEY WORDS

age estimation, skull, sternum, aspartic acid racemization, Thailand

INTRODUCTION

Identifying the biological profile is an important step for the identification of unknown deceased remains. A biological profile helps to reduce the numbers of possible missing persons before matching with unknown deceased, precisely estimated age will reduce the time and cost of identifying an unknown deceased person¹. At present, age estimation by using the aspartic amino acid racemization (AAR) technique from dentine can estimate the chronological age within plus or minus three years of error range². However, forensic cases where skeletal and part of body remains, and teeth may not have been found or may not be well preserved enough to estimate the age by using the AAR technique, other organs such as cartilage and bone may be required.

Even though, bone is a tissue with a low turnover rate and it is not perfect for age estimation by using the AAR method, but there are many reports of age estimation by using the AAR technique from bone³⁵. The most accurate method from bone is analysis of AAR from purified osteocalcin⁶ but this method is complicated and may not be suitable for forensic cases. AAR technique by using the total amino acid of bone is not too complicated and more suitable for routine forensic work⁷. However, this method has a lower accuracy than using osteocalcin and the accuracy of predicted age depends on the type of bone³⁵.

Some reports of age estimation by using AAR from total amino acid of several types of bone such as skull, femur, manubrium, lumbar spine and coccyx, in which the D/L ratio of skull, femur and sternum showed a high correlation with chronological age⁸¹³; thus these bones may be suitable for forensic application. For the femur, when comparing between male and female samples, the correlation of the D/L ratio of male samples showed a higher correlation than females⁸¹². A previous study of age estimation by using the total amino acid from skull and sternum bones reported only male samples and the sample size was quite small. Moreover, the sources of those samples were collected from donated bodies for medical education, which were embalmed¹, so this may not reflect forensic cases.

Moreover, no study of age estimation by using AAR from the total
amino acid of skull and sternum bones in the Thai population and differences of race, genetics, nutrition and environment may affect the rate of aspartic amino acid racemization 13-15). Therefore, the aim of this study was to evaluate age estimation methods using AAR from the total amino acid of skull and sternum bones in a Thai population.

MATERIALS AND METHODS

Reagents and Standard Substances

Standard Aspartic acid (D and L), o-Phthaldialdehyde (OPA) and Nacetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, USA). HPLC grade methanol and other reagents of analytical reagent grade were used for analysis.

Sample Collection and Preparation

Bone samples were collected from autopsy cases from the Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University and bodies that were donated for educational purposes to the Department of Anatomy during June 2016 to April 2017. All of the samples were collected before being embalmed. This study was approved by the Research Ethics Committee of the Faculty of Medicine, Chiang Mai University.

Forty pieces of temporal bone (os temporalis) and sternum (manubro-sternal) were sampled, and approximately one square centimeter of each of the sites of bone were collected. There were 20 male and 20 female samples. Ages ranged from 16 to 87 years, with the mean ages of male and female samples of 49.75 ± 19.72 and 50.75 ± 17.92 years, respectively.

Each bone site was polished to remove the spongy bone, and soft tissue. Then the sample was subsequently washed three times using water and ethanol in an ultrasonicator. Samples were dried at room temperature before pulverization in a freezing mill.

D and L Aspartic Acid Analysis

Demineralization and hydrolysis were performed according to method of Benešová et al. Briefly 10 mg of samples were demineralized by Na2EDTA solution (0.5 M, pH 7.4 adjusted with 1 M NaOH) and NaN3 (0.05 mM) in a micro vial. Then samples were shaken intensively for two hours and centrifuged, after which the supernatant was discarded.

The remaining isolated collagen was transferred to a hydrolyzing tube and 300 μl of 6M hydrochloric acid was added. Then the tubes were flame sealed and heated at 100℃ for six hours. Afterward samples were evaporated to dryness. All samples were prepared in duplicate.

Derivatization was done according to previously described procedures, prepared by dissolving OPA (5.5 mg) in methanol (420 μl) and adding NAC (13.4 mg). The total volume of reagent was adjusted to 10 ml with 0.4 M sodium borate buffer (pH 9.4). The reagent stock solution was stored at 4℃. The hydrolyzed samples were dissolved in 0.1 M hydrochloric acid (1 mL) and diluted ten times prior to derivatization. Dilution sample (100 μl) was mixed with OPA-NAC reagent (200 μl) for five minutes. Sodium phosphate buffer (pH 7.5, 200 μl, 0.3 M) was added, and the mixture was incubated for five minutes. The OPA-NAC-amino acid derivatives (15 μl) were injected into an HPLC system. All samples were analyzed in duplicate.

The high-performance liquid chromatography system and method were according to Monum et al. The mobile phase consisting of A: 30 mM sodium phosphate buffer, pH 5.5; and B: methanol, at a flow rate of 0.7 ml/min, and the injection volume was 15 μl. A step gradient was used (A 85%; B 15% for three minutes, A 85%; B 15% for eight minutes, and A 85%; B 15% for 13 minutes). The fluorescence detector used an excitation wavelength of 337 nm and an emission wavelength of 442 nm. Standard D and L aspartic acids were used for quality control prior to sample analysis, the peak areas of the chromatogram were obtained.

K: Ln(1+D/L)/(1-D/L), R2 coefficient of determination, R correlation coefficient; SEE: standard error of estimation

Table 1. The age estimation equations of D/L aspartic acid from skull and sternum

<table>
<thead>
<tr>
<th>Bone</th>
<th>Sex</th>
<th>Equation</th>
<th>R2</th>
<th>R</th>
<th>SEE (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporal</td>
<td>M</td>
<td>((K-0.0158)/0.0006)</td>
<td>0.7502</td>
<td>0.8661</td>
<td>12.16</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>((K-0.0262)/0.0003)</td>
<td>0.3133</td>
<td>0.5597</td>
<td>26.38</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>((K-0.0203)/0.0005)</td>
<td>0.5114</td>
<td>0.7151</td>
<td>17.46</td>
</tr>
<tr>
<td>Sternum</td>
<td>M</td>
<td>((K-0.0117)/0.0004)</td>
<td>0.3379</td>
<td>0.5812</td>
<td>25.99</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>((K-0.0072)/0.0003)</td>
<td>0.5093</td>
<td>0.7136</td>
<td>16.86</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>((K-0.0095)/0.0003)</td>
<td>0.2778</td>
<td>0.527</td>
<td>31.52</td>
</tr>
</tbody>
</table>

Figure 1. D/L ratio of aspartic amino acid related to chronological age
were calculated the quantity of D and L aspartic acid.

Data Analysis

Age estimations were calculated from $K = a \times \text{age} + b$, $K = \ln((1+D/L)/(1-D/L))$ in which $K$ referred to the coefficient of racemization, $a$ was the rate constant of racemization of bone, and $b$ was the constant (y-intercept); $a$ and $b$ were determined from the linear regression of $K$ and age\(^4\). Standard errors of estimation were calculated to determine the accuracy of the equation. These data were analyzed using the SPSS software package (SPSS for Windows, Version 15, Chicago, IL, USA).

RESULTS

The retention time of D asp-OPA-NAC and L asp-OPA-NAC were 4.3 minutes and 5.4 minutes respectively and clearly according to previous studies\(^6\). The D/L ratio was slightly increased according to chronological age, but the D/L ratio was scattered and not linear in the middle and older ages, (Figure 1).

The average of D/L ratio of temporal bone was higher than sternum in both sexes as shown in Figure 2. The correlation coefficient of regression equation of temporal bone was higher than the correlation coefficient of regression equation of sternum, in which the correlation coefficient of determination of temporal bone was as 0.7151 and 0.5114 respectively, while the correlation coefficient of sternum was 0.5270 and the coefficient of determination was 0.2778 (Table 1).

The standard error of estimation (SEE) of regression equation from temporal bone was 17.46 years, whereas the SEE of sternum was 31.52 years. The sex-regarding equation indicated the SEE of male samples was 12.16 years, which was lower than female samples of about 14 years. The SEE of male samples of sternum was about nine years higher than the female samples.

DISCUSSION

Age estimation from fragmented bone is still important for routine forensic work. Despite, the AAR method from dentin being the best method for age estimation\(^2\) in forensic cases teeth may not be available as part of body remains. Thus, other bone and cartilage may be needed for age estimation. Previous studies of age estimation by using AAR from total amino acid fraction from the femur, skull and sternum of male samples demonstrated a high correlation between the D/L ratio and chronological age.

The results of this present study indicated the average of D/L ratio and the correlation of D/L ratio and chronological age of skull were higher than the sternum. The reason for this is due to the sternum being thin and fragile. Also, during process of samples preparation, some sternal bones needed more time for polishing and washing to remove soft tissue, when compared to the skull. These lead to loss of free amino acid and the D form of aspartic acid from the sternum samples more than the skull samples\(^3\).

In this study fresh samples were used, and fresh bone has some blood and soft tissue contamination which affected the D and L aspartic analysis\(^5\). The previous study used the bone sample from the embalming bodies, which may have had less contamination tissue. These may cause the correlation of D/L ratio and chronological ages obtained from total amino acid fraction of this study to be lower than previous studies. Moreover, temperature, fixative agent, size of pulverized powder, humidity and pH, protein conformation and pathological conditions also affect aspartic acid racemization\(^4,5,17,20,21\).

The correlation of D/L ratio and age obtained from male skull samples were greater than in females, which according to previous studies that indicated sex was related to the rate of AAR in bone. In addition males demonstrated greater correlation to age than females because females tended to be affected by systemic disease more than males\(^8\). On the contrary, the correlation of D/L ratio and age obtained from male sternum samples were lower than female samples. This may have been caused by the contamination of other proteins and the losing of D during the preparation process, which affected the results of AAR analysis.

Compared to previous studies of other types of bone in male samples, the skull and sternum in this study illustrated a lower correlation coefficient of age estimation equation than femur and coxal bone, but it was higher than rib cartilage and sacral bone\(^3,5\).

Even though the SEE of age estimation equation from the skull was high (> 10 years), it still was better than the Meindl and Lovejoy method which was applied to the Thai population\(^6\). This method will be more beneficial in cases of fragmented skull remains. In addition, this method is not too complicated to apply to forensic cases. However sternal bone may not be proper to use for age estimation by using AAR technique in a forensic context due to a high SEE result.

Because the sample size of this study was relatively small, thus, in routine forensic practice the AAR technique should be used with caution and further study of other bone for the Thai population is necessary.

CONCLUSION

Despite the fact that age estimation method using AAR from the total amino acid fraction of skull samples in a Thai population did not demonstrate a high correlation with chronological age it can be beneficial in the case of a fragmented skull. The sternum, however, may not be suitable for use in a forensic context.

ACKNOWLEDGEMENTS

The authors are grateful to Mrs. Joan Elizabeth Peagam for editing the manuscript. This study was supported by a grant from the Forensic Osteology Research Center, Faculty of Medicine, Chiang Mai University and Faculty of Medicine, Chiang Mai University. Research funding was also provided by the Excellence Center in Osteology Research and Training Center (ORTC).

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