Delayed Separation and the Plasma Amino Acids Arginine and Ornithine*

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ABSTRACT

When collecting blood for amino acid testing, leaving plasma in contact with cells at room temperature lowers the concentration of arginine and raises that of ornithine. This is presumably due to the arginase content of red blood cells. In contrast, the sum of arginine and ornithine is constant over the first hour, and defines a reference interval of 74–148 μmol/L (mean ± 2 SD, n = 20) which is more insensitive to delayed separation. The ratio of arginine to the sum of arginine plus ornithine [arg/(arg + orn)] can be used to estimate the number of specimens not separated promptly. A ratio of 0.74–0.50 (mean ± 2 SD, n = 20) is characteristic of specimens placed on ice and separated promptly, where delayed separation produces lower ratios. Of 91 adult specimens received for plasma amino acid analysis over five months, 35 (38 percent) showed a ratio <0.50 suggestive of delayed processing.

Introduction

Numerous studies have been published on plasma amino acids and the collection factors which affect their measurements.1,2,3,4,5 One of the difficulties when examining amino acid patterns is the fact that more than 20 separate analytes are involved, each with its own analytic and preanalytic variability. In order to cope with this complexity, the present study has concentrated on arginine and ornithine, and the effects seen when blood is not chilled and plasma is not separated promptly from the cellular components of blood. Factors which affect arginine are particularly interesting considering its central role in nitric oxide (NO) metabolism.6

The biochemical relationship of arginine and ornithine is well-known, with both amino acids participating in the urea cycle. Under the influence of the enzyme arginase, arginine is converted to ornithine and urea.7 While a portion of the urea cycle occurs in mitochondria, arginase is a cytosolic enzyme present at highest concentration in hepatocytes. However, arginase is also present at lower concentrations in a wide variety of other cell types, including leukocytes8 and erythrocytes.9 Thus, plasma arginine can be taken up by erythrocytes, converted into ornithine and subsequently released back into the plasma.10 While the physiologic reasons for this process are not understood, the purpose here is to describe the effect this has on the collection of plasma amino acid specimens.

Because the amino acid content of plasma is significantly different from the cells,2,3,10 care-
ful collection of the specimen is needed if the results are to reflect the physiological state of the patient. Ideally, the patient is fasting, blood is drawn into an heparin-containing tube which is mixed gently and placed quickly on ice. The blood is then centrifuged, and the plasma separated promptly while avoiding hemolysis and buffy-coat material containing leukocytes and platelets. However, plasma specimens are often collected and processed under less than ideal conditions. Personnel may be unaware of the preanalytic issues, ice may not be readily available, or centrifugation may be delayed. An additional confounding factor is that the cells of the buffy-coat may be inadvertently included in an attempt to get the last bit of plasma, particularly when blood collection has been difficult.3 The relationship of arginine and ornithine may be useful to identify specimens subjected to delays in separation, and to estimate how often this occurs.

Methods

Amino acids were determined as described elsewhere,11'12 using a method based on automated precolumn o-phthalaldehyde (OPA) derivatization, high performance liquid chromatography (HPLC) and fluorescence detection. Instrumentation consisted of an Hewlett-Packard (Palo Alto, CA) HP1090 liquid chromatograph equipped with an autoinjector and HP1046 fluorescence detector (excitation and emission 230 and 455 nm, respectively). The separation was performed using a reverse-phase HP Hypersil ODS3 column (5 μm, 20 cm x 4.5 mm internal diameter) with gradient elution (mobile phase A: 0.05 M sodium acetate, 0.6 percent tetrahydrofuran and 0.015 percent triethylamine; and mobile phase B: acetonitrile, 0.1 M sodium acetate and methanol in a ratio of 14:4:1).

Specimen preparation consisted of adding 200 μL plasma and 20 μL nortryptiline internal standard to a Millipore (UFC3 L6C, Bedford, MA) centrifuge-type filtration device and centrifuging at 5000 g for 45 minutes at 10°C. The filtrate was transferred to an instrument vial and loaded into a refrigerated sample compartment for automated derivatization and injection onto the column.

Blood was collected by a trained phlebotomist from healthy, adult volunteers in compliance with the ethical standards of this institution. Several different protocols were followed. For the reference interval study, blood was collected into heparinized vacutainers, the tubes placed quickly on ice and the plasma separated within 20 minutes. For the timed studies represented in figure 1, multiple blood specimens were drawn, the time zero specimen placed quickly on ice and the plasma separated within 20 minutes; the remaining specimens were left at room temperature for the specified time before being transferred to ice and separated.

All adult (≥20 years old) specimens for plasma amino acids received at this institution between January 1 and May 20, 1998, were collected (n = 91). The arginine and ornithine concentrations were compiled, and the ratios of arginine to the sum of arginine plus ornithine [arg/(arg + orn)] calculated.

Results

As illustrated in figure 1, the concentration of arginine decreases and that of ornithine increases when plasma remains in contact with cells. In contrast, when specimens were quickly placed on ice, the amino acid concentration did not change significantly even when the plasma remained in contact with the cells for up to three hours (data not shown). Likewise, arginine and ornithine did not show significant change in plasma left at room temperature unless hemolysis was also present.

Figure 1 shows the results from specimens collected from a 25-year-old male; the same phenomena was observed in all specimens examined in a similar fashion (n = 10). Specimens collected in an EDTA anticoagulant as opposed to heparin show an essentially identical pattern in terms of arginine and ornithine. In figure 1, the [arg/(arg + orn)] ratio was 0.70 at time zero (placed immediately on ice), 0.56 at 0.50 hour (that is, half-an-hour elapsing
before being placed on ice), 0.50 at 1 hour, 0.45 at 1.5 hour, 0.42 at 2 hour and 0.40 at 2.5 hour. The initial rate of arginine decline in figure 1 is about 0.5 \(\mu\text{mol/L}\) per minute at the initial arginine concentration of 88.6 \(\mu\text{mol/L}\).

For 20 carefully collected specimens from healthy adults (11 males and nine females), the average concentration of arginine was 69 \(\mu\text{mol/L}\) (SD 13 \(\mu\text{mol/L}\), skew -0.31), that of ornithine was 42 \(\mu\text{mol/L}\) (SD 11 \(\mu\text{mol/L}\), skew 1.09), and the sum of arginine and ornithine was 111 \(\mu\text{mol/L}\) (SD 19 \(\mu\text{mol/L}\), skew 0.33). The ratio \([\text{arg} / (\text{arg} + \text{orn})]\) was 0.62 (SD 0.06, skew -0.35). A portion of this information is summarized in table I.

Ninety-one adult specimens were received for plasma amino acid analysis in the clinical reference laboratory at this institution between January 1 and May 21, 1998. The arginine and ornithine concentrations were examined, and the results summarized in table I. For this population, the average concentration of arginine was 70 \(\mu\text{mol/L}\) (SD 34), the average ornithine was 71 \(\mu\text{mol/L}\) (SD 31) and the average ratio \([\text{arg} / (\text{arg} + \text{orn})]\) was 0.49 (SD 0.13). The range of the ratio was 0.70–0.09, with 35 specimens showing a ratio <0.50, 19 specimens < 0.40, 7 < 0.30, 3 < 0.20 and 1 < 0.10.

**Discussion**

Figure 1 shows an example of the typical time course associated with delayed separation of plasma from cells when left at room tem-

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**FIGURE 1.** The changes in plasma arginine and ornithine associated with delayed separation of plasma from cells. Heparinized blood was collected from a 25-year-old male, the time zero specimen was placed immediately on ice, whereas the remaining specimens were left at room temperature for the specified time prior to being placed on ice. Separation of plasma occurred within 20 minutes of chilling.

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**TABLE I**

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>(n)</th>
<th>Arginine(^1) (\mu\text{mol/L}\ ± SD.)</th>
<th>Ornithine(^1) (\mu\text{mol/L}\ ± SD.)</th>
<th>Ratio(^2)</th>
<th>Ratio &lt; 0.50(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ideal(^4)</td>
<td>20</td>
<td>69 ± 13</td>
<td>42 ± 11</td>
<td>0.62 ± 0.06</td>
<td>0</td>
</tr>
<tr>
<td>Routine(^5)</td>
<td>91</td>
<td>70 ± 34</td>
<td>71 ± 31</td>
<td>0.49 ± 0.13</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^1\)\(\mu\text{mol/L}\ ± SD.\)

\(^2\)Ratio of \([\text{arg} / (\text{arg} + \text{orn})]\) ± SD.

\(^3\)A ratio < 0.50 suggests a significant delay in the separation of plasma from cells.

\(^4\)Placed quickly on ice and separated within 20 minutes.

\(^5\)Patient specimens received without knowledge of processing.
While the arginine concentration decreases and that of ornithine increases, the sum of the two remains relatively constant, particularly during the first hour. The authors are not aware of previous publications describing this phenomena. Schaefer et al. noted the changes which occurred with arginine and ornithine after six hours of delayed separation, although they did not discuss the time-course of the process. Arginine and ornithine do not change significantly in isolated plasma or when blood is placed promptly on ice. However, as has been described previously, collection of hemolyzed plasma is associated with a decline in arginine and an increase in ornithine.

Using carefully collected plasma from 20 healthy adults, the following reference intervals (mean ± 2 SD) were calculated: Arginine 44–94 µmol/L and ornithine 21–63 µmol/L. Amino acid testing is relatively complex, expensive and time-consuming, so many reference interval studies are based on small populations (table II). The small populations contribute to the differences seen in published intervals, as do variations in analytic methods, and preanalytic variables such as described here. The authors suspect that larger populations for amino acid reference interval studies are unlikely to resolve discrepancies until the complexities of preanalytic variation are more completely characterized.

In a comprehensive study of amino acid reference intervals in elderly subjects, Prior et al. discuss differences seen in arginine and ornithine in various elderly subgroups. Although these authors carefully examined a wide variety of physiologic variables, they did not specifically address preanalytic issues. It is our hypothesis that some of the subpopulation differences seen by these authors are due to preanalytic collection problems, namely, that specimens from different locations were collected and processed differently. Two points are worth emphasizing: 1) A wide variety of factors influence amino acid results, and 2) preanalytic factors are often ignored.

In the present study the distribution of arginine is negatively skewed (−0.31), that of ornithine is positively skewed (1.10) and the sum of the two is intermediate (0.33). Scriver et al. describe the distributions as essentially normal, although their calculations do show a small degree of skewing: −0.031 for arginine and 0.13 for ornithine. Their specimen collection protocol described very prompt separation of plasma, although it did not include placing specimens quickly on ice. This factor would tend to reduce the average concentration of arginine, thereby also reducing the negative skew in the distribution; likewise, the average concentration of ornithine would be increased, reducing the positive skew. In contrast, the population distributions are more skewed when specimens are placed promptly on ice, at least in this relatively small study.

Defining a reference interval in terms of the sum of arginine and ornithine offers some advantages. A combined range emphasizes the intrinsic biochemical relationship between these two amino acids, and is also less sensitive to the effects of delayed plasma separation. The present study found a combined reference interval of 74–148 µmol/L (mean ± 2 SD, n = 20). This interval is not identical to that which would be calculated by adding the two individual reference intervals together (ie, arginine 44–94 µmol/L + ornithine 21–63 µmol/L = combined 65–157 µmol/L). This is due to the fact that the distributions are not Gaussian.

The relationship of arginine and ornithine can be used to estimate the time which elapsed between the collection of blood and the subsequent separation of plasma. The sum of argi-

TABLE II

Comparison of Reference Intervals for Plasma Arginine and Ornithine

<table>
<thead>
<tr>
<th>Arginine (µmol/L)</th>
<th>Ornithine (µmol/L)</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>44 – 94</td>
<td>21 – 63</td>
<td>20</td>
<td>Present study</td>
</tr>
<tr>
<td>48 – 116</td>
<td>30 – 86</td>
<td>20</td>
<td>Perry and Hansen</td>
</tr>
<tr>
<td>54 – 134</td>
<td>32 – 100</td>
<td>80</td>
<td>Scriver et al</td>
</tr>
<tr>
<td>30 – 114</td>
<td>21 – 95</td>
<td>16</td>
<td>Zhang and Pang</td>
</tr>
<tr>
<td>60 – 120</td>
<td>21 – 93</td>
<td>44</td>
<td>Terrlink et al</td>
</tr>
<tr>
<td>51 – 111</td>
<td>43 – 103</td>
<td>44</td>
<td>Fekkes et al</td>
</tr>
<tr>
<td>40 – 120</td>
<td>23 – 87</td>
<td>100</td>
<td>Le Boucher et al</td>
</tr>
</tbody>
</table>

*aMean ± 2 SD in healthy adults.*
nine and ornithine is relatively constant in any given individual; therefore this value was placed in the denominator. In 20 carefully collected specimens, the ratio of arginine to the sum of arginine plus ornithine \( \frac{\text{arg}}{\text{arg} + \text{orn}} \) was 0.74–0.50 (mean ± 2 SD). When specimen processing was delayed, the ratio was lower. By assuming that all specimens which show a ratio <0.50 have not been placed on ice and separated promptly, one can estimate the number which have undergone a significant delay in processing—although there are some obvious limitations. First, this ratio was evaluated only in a small number of healthy adults. Second, a variety of disease processes could potentially affect the two amino acids; presumably, such diseases will be less frequent than specimen processing problems. In addition, the ratio can be relatively insensitive, with values <0.50 seen only after significant delays. In figure 1, for example, the ratio at time zero is 0.70, and does not drop below 0.50 until more than one hour after collection.

The assumption described above was applied to a population of 91 adult specimens received for plasma amino acid analysis at this institution. Table I summarizes the differences seen in these routinely collected specimens versus those processed under ideal conditions (ie, placed quickly on ice and separated within 20 minutes). For the 91 routine specimens, the arginine-ornithine ratio ranged from 0.70–0.09 (mean ± 2 SD), with 35 specimens (38 percent) <0.50 and 19 specimens (21 percent) <0.40. In other words, 38 percent of specimens showed characteristics suggestive of delayed separation and 21 percent suggested more extreme delays.

Since serum amino acids show a considerable increase in variability, serum should never be substituted for plasma.\(^1,2\) The collection of plasma is more complicated than the collection of serum and is probably an important factor in the high incidence of delayed separations. Just as with serum, improper collection of plasma makes the interpretation of results more difficult and obscures potentially important information.\(^1,3,4,5\) The relationship between arginine and ornithine can be used to provide an objective assessment of the quality of plasma specimens and may prove to be a useful tool with which to improve the quality of specimen collection.

**Conclusion**

The concentration of plasma arginine decreases and that of ornithine increases when the separation of plasma from cells is delayed, where the sum of the two is less sensitive to delays in separation. Defining a reference interval (mean ± 2 SD, n = 20) for the sum of arginine plus ornithine (74–148 \( \mu \text{mol/L} \)) is less affected by delays in plasma separation. The ratio of arginine to the sum of arginine plus ornithine \( \frac{\text{arg}}{\text{arg} + \text{orn}} \) in carefully collected specimens is 0.74–0.50 (mean ± 2 SD, n = 20). By assuming that all plasma specimens with a ratio <0.50 are due to delayed separation, the incidence of delayed separation can be estimated. Using this approach, specimens received for amino acid analysis show characteristics of delayed separation in 38 percent of those examined. Although the limits of this approach have not been fully characterized, the ability to identify specimens suspicious for delayed separation may be useful to identify artifacts which can interfere with interpretation, and to provide feedback to those involved in the specimen collection process.

**References**

PLASMA AMINO ACIDS ARGININE AND ORNITHINE