Ornithine levels in human serum after oral dosing measured by a colorimetric assay

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A colorimetric method for analysis of ornithine in human serum and crystalline amino acid mixtures was developed, utilizing simple reagents, equipment, and techniques suitable for smaller clinical laboratories. Serum samples or amino acid mixtures were incubated with ornithine carbamyl transferase to quantitatively convert ornithine to citrulline. Urease addition removed interfering substances. After deproteinization, citrulline was measured by modified diacetyl monoxime/phenazone color formation. Blanks accounted for endogenous citrulline levels and nonspecific interferences. This assay was used to follow levels of serum ornithine in weight lifters after oral dosing with 40, 100, and 170 mg ornithine/kg body weight. A dose-dependent increase in levels of serum ornithine was found, leading to a 400% increase in serum levels 90 min after ingestion of 170 mg/kg. The present colorimetric assay compared favorably to other more complex, laborious, and expensive methods, enabling a wider range of laboratories to study ornithine.

Keywords: ornithine; citrulline; ornithine assay; human subjects; weight lifters

Introduction

Ornithine, a dispensable amino acid found in negligible amounts from dietary sources, is involved in the urea cycle, polyamine synthesis, and arginine metabolism.[1] Ornithine can evoke somatotropin release in humans after intravenous loading,[2,3] a finding that has spurred many weight lifters and dieters to consume amino acid supplements containing ornithine in an attempt to stimulate anabolism via release of somatotropin.[4,5] The recent finding of increased serum somatotropin levels after ingestion of a range of oral doses of ornithine necessitated verification of association of increased serum ornithine levels with increased serum somatotropin levels.[6] Also, verification of label claims of ornithine amounts in dietary supplements was an additional need. The technique of choice for measuring ornithine, an amino acid analyzer, was unavailable because of cost and logistical constraints.

Analysis of ornithine levels in biological samples usually is accomplished by ion-exchange chromatography (amino acid analyzers)[7-10] or by counting radioactive products after enzymatic treatments with ornithine decarboxylase.[11] For smaller laboratories that do not possess resources to utilize chromatographic equipment or radioactive compounds, there are few options available for estimation of ornithine levels. Thin-layer chromatographic procedures are laborious and subject to interferences.[12] One colorimetric method suffers from interferences with lysine.[13,14] Other methods require equipment or reagents uncommon to most laboratories.[15,16]

The assay described in this report combined modifications of several methods for analysis of ornithine carbamyl transferase (OCT).[14,17-21] This assay possesses sufficient sensitivity and accuracy for determination of ornithine in samples of human serum or mixtures of amino acids. Almost all educational or clinical laboratories can perform this assay using commercially available reagents and basic equipment.

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Methods and materials

Subjects

Permission to utilize human subjects was granted by the Human Review Boards of University of Houston and University of Texas Health Science Center at Houston. Active, trained weight lifters were recruited from local gyms. Normal subjects were employees of Biotics Research Corporation. Three female weight lifters (ages: 34.3 ± 5.1 years; weight: 58.2 ± 7.6 kg; height: 166 ± 8 cm; mean ± SD) and ten male weight lifters (ages: 28.1 ± 7.0 years; weight: 82.5 ± 17.4 kg; height: 172 ± 8 cm) were given 40, 100, or 170 mg ornithine/kg at 8:00 AM after an overnight fast. Serum was obtained at 0, 45, and 90 min after ingestion of ornithine and stored frozen at −40°C until assay.[6]

Ornithine estimation

Serum samples were reacted with excesses of ornithine carbamyl transferase (OCT), urease, and carbamyl phosphate to convert ornithine quantitatively to citrulline and to remove urea, which interferes with later citrulline analysis. Blanks were run for each sample to account for endogenous levels of citrulline and other possible interfering substances, as well as nonspecific formation of citrulline. After reaction with enzymes, samples were deproteinized with trichloracetic acid (TCA), and the supernatant analyzed for citrulline content. A modification of the diacetyl monoxime/phenazone method described by others[14,17-21] was used. Ornithine concentration in samples was determined from a standard curve of ornithine treated identically as samples. A standard curve was determined with each batch.

Enzymatic reaction procedure

Two glass, 13 × 100 mm test tubes were labeled for each sample (Blank and Test). To each Blank tube, 0.6 ml of triethanolamine (TEA) buffer (0.4 M TEA, pH 7.7) was added. To each Test tube, 0.5 ml of TEA buffer was added. To each Blank and Test tube, 0.1 ml carbamyl phosphate solution (50 mM dilithium carbamyl phosphate in TEA buffer, prepared fresh before each use), and 0.1 ml urease solution (100 U/ml in TEA buffer, stored in refrigerator) were added. To each Test tube, 0.1 ml of OCT solution (20 U OCT/ml in TEA buffer, stored in refrigerator) was added. A timer was started and 0.2 ml of sample or standard was added to appropriate Blank and Test tubes. Tube contents were mixed and placed in a 37° C water bath for 20 min. After 20 min, 1.0 ml of cold 10% TCA (wt/vol) was added to each Blank and Test tube in the same order that samples were added. Each tube was centrifuged (approximately 1000 × *g* for 10 min) to pellet the resulting precipitate. After centrifugation, TCA supernatants may be stored before subsequent citrulline assay.

Citrulline assay

A modification of several methods was used to estimate citrulline (and thus ornithine) levels in TCA supernatants.[12,15-19] Diacetyl monoxime (0.4% in 7.5% sodium chloride) was stored in a dark bottle at room temperature and was stable for six months. Acidic phenazone/ferric ammonium sulfate solution was prepared by combining 0.37 g phenazone, 0.25 g $FeNH_4(SO_4)_2 \cdot 12H_2O$, 25 ml concentrated sulfuric acid, and 25 ml of 85% phosphoric acid, and with careful addition of water for a final volume of 100 ml. Initially, this solution may be cloudy, but this has no effect on the outcome of assays.

To 16 × 125 mm glass screw-top tubes, 0.5 ml of TCA supernatant was added. Color reagent was prepared fresh for each batch of determinations and used immediately. Color reagent was prepared by mixing one volume of diacetyl monoxime solution with 2 volumes of acidic phenazone/ferric ammonium sulfate solution in a dark bottle. A timer was set for 15 min, then 3.0 ml of color reagent was added to a tube, loosely covered, then placed immediately in a boiling water bath. The process was repeated for the next tube, and so on for each tube. The bath was covered when all samples were emplaced. After 15 min, the bath was uncovered, then the first tube put into the bath was removed and placed in an ice-water bath in the same sequence they were added. When all tubes were placed in the ice bath, the first tube was removed and placed in a room temperature water bath, followed by sequential addition of the remainder of tubes. In this manner, each tube had equivalent exposure to heat and light. One must be sure to perform different batches of citrulline assays under the same lighting conditions for least between-batch variation. Absorbance at 464 nm was measured for each tube in the same sequence as before, with distilled water as zero. Covered cuvettes were preferred because the color reagant is highly acidic and corrosive to spectrophotometers.

Standard curve and reagents

TEA, dilithium carbamyl phosphate, OCT, urease, L-citrulline, phenazone (antipyrine), and diacetyl monoxime were obtained from Sigma Chemical Company (St. Louis, MO). L-ornithine hydrochloride was obtained from Tanabe Company (Tokyo, Japan). All other chemicals used were of analytical reagent grade. Mixtures of free amino acids were prepared and supplied by Biotics Research Corporation.

L-ornithine hydrochloride was dissolved in TEA buffer at concentrations ranging from 0–2965 nmol/ml to determine the range of concentrations for linearity of the standard curve. Since this assay was employed to measure serum levels of ornithine after oral loading, a standard curve with concentrations ranging from 0–1000 nmol/ml was routinely performed. Otherwise, a standard curve ranging from 0–200 nmol/ml would suffice for ornithine determinations in normal serum samples. Standards were processed identically as samples. Two dietary supplement products containing mixtures of free amino acids, including known amounts of both citrulline and ornithine, were used as controls. Products were dissolved at concentrations of

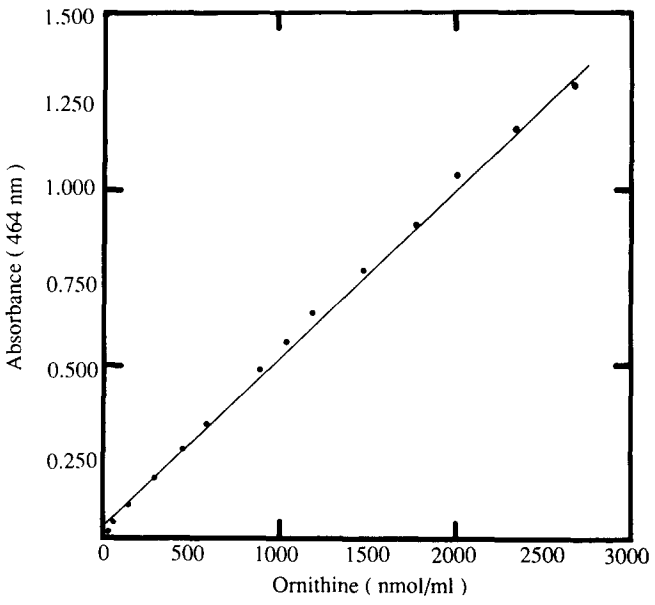

Figure 1 Standard curve for determination of linearity of colorimetric assay for ornithine. Ornithine hydrochloride was dissolved in triethanolamine buffer at concentrations ranging from 0–2965 nmol/ml and reacted with carbamyl-phosphate, ornithine carbamyltransferase, and urease. After addition of 10% trichloracetic acid and centrifugation, diacetyl monoxime/phenazone color reagent was added to the clear supernatant and boiled. Absorbance at 464 nm was determined. Simple linear regression analysis yielded the equation $Y = 0.025 + 0.490X$. For analysis of serum ornithine levels after oral loading with ornithine, a standard curve ranging from 0–1000 nmol/ml was employed routinely. For determination of serum ornithine levels in normal human serum samples, a standard curve ranging from 0–200 nmol/ml would suffice.

1.0 mg/ml in TEA buffer (ornithine control) or 10% TCA (citrulline control).

Statistical analysis utilized Student's *t* test or analysis of variance (ANOVA).

Results

Assay characteristics

Under the specified assay conditions, concentrations of 18–2700 nmol/ml of ornithine produced a linear standard curve (*Figure 1*). Two amino acid mixtures used as controls for the citrulline assay contained 21.2 and 14.6 nmol/ml of citrulline, respectively. Assayed values for citrulline were 22.5 (106%) and 14.5 (99.6%) nmol/ml. These two mixtures also contained ornithine at concentrations of 66 and 454 nmol/ml, respectively. Assayed values for ornithine were 56.5 and 396 nmol/ml (85.5 and 87.2%, respectively) of actual values. Ornithine at concentrations up to 600,000 nmol/ml in 10% TCA did not produce color in the citrulline assay.

Recovery of L-ornithine added to a human serum sample was 98.1% ($n = 10$) at a concentration of 892 nmol/ml. Interassay coefficients of variation (CV) for replicate analyses of ornithine ($n = 10$) of normal (156 ± 8.4 nmol/ml) and elevated (315 ± 17 nmol/ml) pooled serum controls were both 5.4%. Intra-assay CV for replicate analyses ($n = 10$) of two different serum samples were 5.1 and 4.0%.

Levels of ornithine in human serum

Table 1 exhibits serum levels of ornithine in normal subjects and weight lifters. *Figure 2* shows the levels of ornithine found in weight lifters before and after oral dosing with 40, 100, or 170 mg/kg of L-ornithine

Table 1 Comparative values for human serum levels of ornithine by different assay methods

Subjects	Methods of analysis	Number of subjects (female + male)	Serum ornithine (nmol/ml ± SD)	Reference
Weight lifters[a]	Colorimetric	3 + 10	125 ± 33	—[b]
Female weight lifters[c]	Colorimetric	3	106 ± 22	—[b]
Male weight lifters[d]	Colorimetric	10	132 ± 18	—[b]
Normal adults[e]	Colorimetric	3	92 ± 8.9[f]	—[b]
Normal adults	Enzymatic	3 + 10	76 ± 11	11
Normal adults	Amino acid analyzer[g]	8	100 ± 12	18
Normal adults	Amino acid analyzer	2 + 5	85 ± 27	20
Normal adults	Amino acid analyzer	5	77 ± 28	5
Normal females	Amino acid analyzer	7	61 ± 6.6	19
Normal females	Amino acid analyzer	35	60 ± 17	5
Normal females	Amino acid analyzer	101	54 ± 18	21
Normal males	Amino acid analyzer	12	75 ± 6.6	19
Normal males	Amino acid analyzer	37	85 ± 24	5
Normal males	Amino acid analyzer	90	65 ± 18	21

[a] Two or three separate samples from each subject, collected one week apart, were assayed, for a total of 36 determinations.
[b] This study.
[c] Nine total determinations were measured.
[d] Twenty-seven total determinations were measured.
[e] One male, one female, and a serum pool from eight normal males were assayed thrice each for a total of nine determinations.
[f] Significantly different from weight lifters by Student's two-tailed *t* test ($t = 2.993$, $P < 0.005$).
[g] Ion-exchange chromatography as described in references 7–10, 22–25.

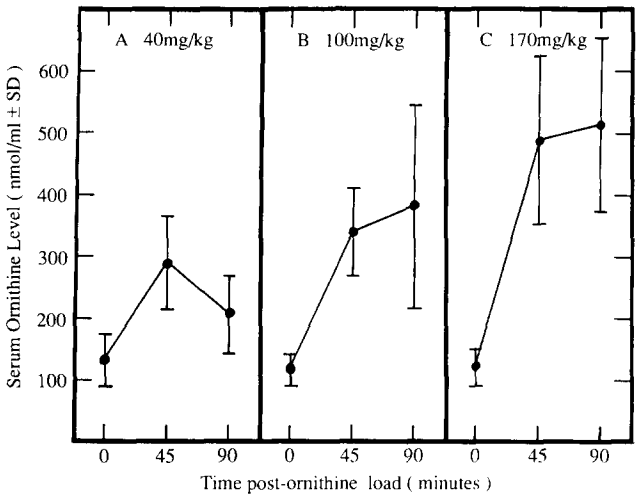

Figure 2 Changes in levels of ornithine (mean ± Standard Deviation) in serum of twelve weight lifters (three females, nine males) after oral doses of 40 (A), 100 (B), or 170 (C) mg ornithine/kg body weight. Serum changes in levels of ornithine were significantly different from starting values at 45 and 90 min for each dose by ANOVA ($P > 0.001$).

hydrochloride. A dose-dependent increase in serum ornithine levels was found.

Discussion

Analysis of powdered amino acid mixtures with low (1.1% wt/wt) or high (7.7% wt/wt) ornithine contents indicated that 85–87% of ornithine was converted to citrulline by the enzymatic half of the assay. Low recovery of ornithine from free amino acid products may be due to inefficient enzymatic conversion of ornithine to citrulline, or to incomplete mixing of the product itself. Since ornithine standards and serum samples were treated identically, possible incomplete conversion of ornithine to citrulline would not affect results.

The second half of the assay (citrulline estimation) showed complete recovery of citrulline (99–106%), as shown by powdered amino acid samples containing citrulline calculated from a genuine citrulline standard curve.

Compared to other methods,[9,15,22-25] the present assay yields similar results for normal adults (*Table 1*). Cost restraints prevented analysis of replicate samples by other methods; therefore, historical controls are compared. Weight lifters exhibited significantly higher levels of serum ornithine than normal adults ($t = 2.993$, $P < 0.005$). Similar to trends seen by others in normal subjects, a trend for lower levels in females was also seen for weight lifters, although the difference was not significant ($t = 1.000$, $P > 0.20$) (*Table 1*).

Possible explanations for the apparent elevated level of ornithine in weight lifters include consumption of high-protein diets, consumption of amino acid supplements containing ornithine, an enhanced flux of the urea cycle (or other metabolic pathways requiring ornithine), or liver damage allowing urea cycle components to appear in the bloodstream.

Dietary sources of ornithine are negligible at best, since ornithine is not incorporated into proteins. Thus, even if weight lifters consume 100–200 g of protein daily, this amount of protein contains negligible amounts of ornithine. It remains a possibility that excess availability of amino acids to organs may lead to higher than normal levels of ornithine synthesis.

Only two subjects regularly consumed large amounts of ornithine daily (750 mg). Both were female, and exhibited values slightly lower than the mean for other subjects. Five other subjects consumed amino acid supplements supplying less than 500 mg of ornithine daily. Their values of serum ornithine were not different from the other eight subjects that did not consume supplemental ornithine. Thus, ingestion of ornithine from dietary supplements did not account for high levels of serum ornithine in our study population.

Ammonia is produced primarily by fast twitch muscle fibers during intense exercise.[26] Our subjects routinely engaged in exhaustive resistance training, which recruits mostly fast twitch muscle fibers.[26] It is possible that weight lifters may adapt to exercise-induced, elevated ammonia levels by increasing the capacity of the urea cycle, much like antioxidant enzyme levels increase in response to the free radical burden of exercise.[26] Increased capacity of the urea cycle may account for increased serum levels of ornithine at rest.

Liver damage was not apparent in any subject, as exhibited by normal values for indicators of liver function for each subject after analysis by multi-channel blood chemistry profiles. Thus, an explanation for increased serum ornithine levels in weight lifters awaits further study.

In detailed comparisons to other methods of ornithine analysis in human serum, the present assay offers several advantages. First, no expensive equipment or reagents, other than a spectrophotometer, are required, unlike ion-exchange chromatography by amino acid analyzers.[7-10,22-25] Second, the present assay makes use of commercially obtainable enzymes, unlike another enzymatic method that coupled ornithine aminotransferase (EC 2.6.1.13) and delta-1-pyrroline-5-carboxylate reductase (EC 1.5.1.2), which have no commercial sources.[15] Third, the present assay appears free from major interferences from lysine found in biological samples that confuse colorimetric assays with Chinard's ninhydrin reagent.[13,14] Fourth, under the conditions of the assay for citrulline described in this paper, we did not find an extreme dependence on heat and lighting conditions as noted by other investigators.[21] Rather, we found that color development was stable for one hour when exposed to normal room lighting conditions, similar to other investigators.[20] It may be necessary for each laboratory to evaluate the effect of their lighting conditions on the citrulline colorimetric portion of this assay. Finally, when compared to the only previous report using OCT coupled with citrulline analysis for estimation of ornithine,[14] the present assay did not exhibit high

blanks. The previous report employed liver acetone powders for an OCT source, leading to unacceptably high blanks.[14] We avoided this problem by using purified enzymes and urease to reduce blank levels to minimal values.

Thus, the assay described in this paper is a unique method for ornithine determination in human serum suitable for smaller laboratories that do not possess expensive equipment or that do not have budgets allowing payment for outside testing (i.e., educational and clinical laboratories).

Abbreviations

OCT ornithine carbamyl transferase (EC 2.1.3.3)
TEA triethanolamine
TCA trichloracetic acid
CV coefficient of variation

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