



# Impact of suitable control on a uniform interpretation of units for arginase assay

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## ABSTRACT

The arginase catalyzes the conversion of arginine into ornithine and urea. The activity of arginase serves as a critical diagnostic marker for several pathophysiological conditions. However, a specific, sensitive, and universal assay system for arginase with suitable control is elusive. Mostly amount of either urea or ornithine is estimated but an interpretation of the activity of arginase needs to be re-evaluated considering the endogenous level and influence of the substrate. This report; has been intended to evaluate methods of arginase assay and suitable controls. A conversion factor has been suggested for uniform interpretation of units for arginase assay.

## 1. Introduction

The arginase critically regulates the homeostasis of arginine, ornithine, and urea [1], production of NO in the inflammation and neurological complication [2], cell proliferation [3], vascular abnormalities [4], diabetes [5], cardiovascular diseases [6], and biology of tumor or cancer [7]. The activity of arginase serves as a diagnostic marker which is measured by colorimetric estimation of either arginine (substrate) or ornithine and urea (products) [8]. Preferably, ornithine is estimated either by the method of Chinard [9] or by Archibald [10], and the urea is determined by the direct method of Diacetyl Monooxime [11] or by the indirect methods which convert urea into ammonia and detection of ammonia either by the Nessler's reagent or by the Berthelot's method. Various improvements have been made over time to overcome limitations of the reaction between the component, prolonged boiling, low sensitivity, and use of corrosive materials, but the uniform interpretation of units for arginase assay needs attention. The results from the available methods describe a trend of specific assay but the uniform presentation of the units for arginase assay is elusive due to lack of suitable controls in assay conditions [12,13]. This report presents an evaluation of known methods of urea and ornithine estimation and suggests a conversion factor to derive a uniform measure of arginase activity. (see Fig. 1)

## 2. Material and methods

### 2.1. Animals and materials

The young (4 weeks) mice (*Mus musculus*) of AKR strain were used for experiments. The animals were maintained with standard mice feed and drinking water at  $25 \pm 2$  °C in the animal house facility of the department as per guidelines of the Institutional Animal Ethical Committee. Animals were sacrificed and the liver was dissected out. The liver tissue of *Mus musculus* was used to prepare enzyme extract as described earlier [14]. All chemicals were used of analytical grade purchased from Sigma, Himedia, SRL, and other indigenous sources.

### 2.2. Enzyme assays

The reaction mixture consisting of 25 mM sodium glycinate buffer (pH 9.5), 2.5 mM MnCl<sub>2</sub>, 25 mM L-arginine, and suitably diluted enzyme extract, in a total volume of 2 ml, was incubated for 15 min at 30 °C. The reaction was terminated by adding 10% Perchloric acid. The urea and ornithine were determined as per the methods of Brown and Cohen [8]. In the method of ornithine estimation (E1) by Chinard [9], 1 ml of the supernatant and 2 ml of 2% ninhydrin solution were boiled for 25min followed by cooling at room temperature and the absorbance was taken at 505 nm. The amount of urea is presented in 1 μmol/ml. In the Chemical method of Berthelot's (E2) 10 μl of the supernatant was mixed with 1 ml of the enzyme (Urease) and incubated for 5 min at 37 °C. After that, 1 ml of chromogenic substance was added which gives yellow to

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green color depending on the amount of urea at 600 nm. The amount of urea is calculated by the  $(OD \text{ of test}/OD \text{ of standard}) \times 40 \times \text{dilution factor} \times 0.36$ . The amount of urea is presented in  $1 \mu\text{mol/ml}$ . The direct method of urea determination (E3) by Moore and Kauffman [11] was adapted in which 1 ml of the supernatant was mixed with 2.5 ml of the acid mixture ( $\text{H}_3\text{PO}_4 + \text{H}_2\text{SO}_4 + \text{water}$ , in 3:1:4) followed by the addition of 0.25 ml of 3% DAM. The mixture was boiled for 30 min and then cooled at room temperature and absorbance was taken at 478 nm. The amount of urea is presented as  $1 \mu\text{mol/ml}$ . One unit of arginase activity was defined as the amount of enzyme, which produced  $1 \mu\text{mole}$  of urea or ornithine produced/min at  $30^\circ\text{C}$ . Since the use of control and blank is poorly described in almost all reports, various possible controls were used (buffer, buffer +  $\text{MnCl}_2$ , buffer +  $\text{MnCl}_2$  + tissue (mice liver), buffer +  $\text{MnCl}_2$  + Arginine, as different blank conditions with a complete

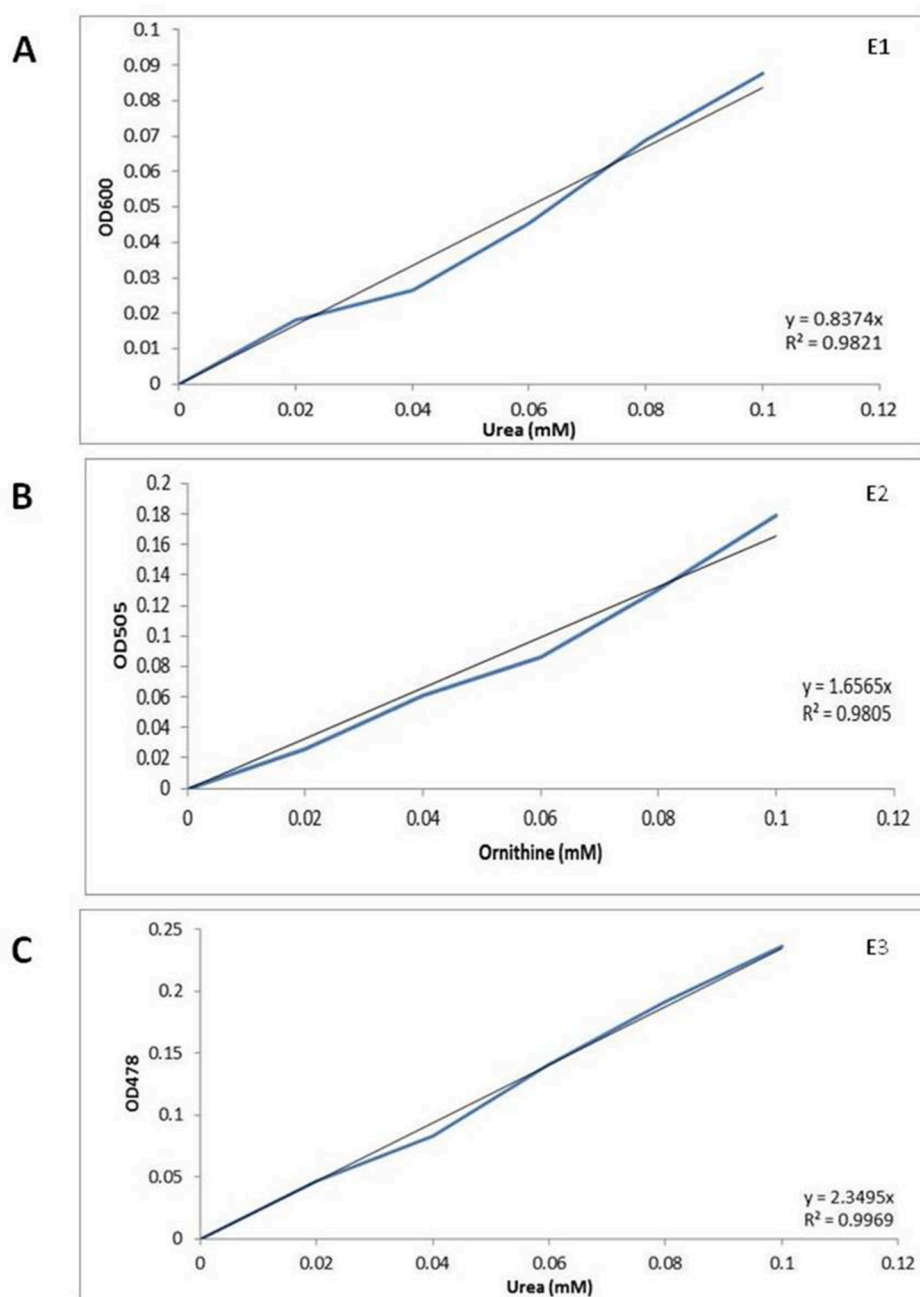
reaction mixture of buffer +  $\text{MnCl}_2$  + arginine + tissue extract. The level of endogenous urea was also considered and estimated in all reactions.

### 2.3. Statistical analysis

All experiments were carried out in triplicate and the enzyme activities and Integrated Density Value shown in mean  $\pm$  S.D. The significance of mean was analyzed by the Student's t-test and  $p < 0.05$  taken as significance level and denoted by \* in this manuscript.

## 3. Result and discussion

The analysis of results using different standard curves (Fig 1 and Table 1) from a range of urea (0.02–1 mM) and ornithine (0.02–1 mM)



**Fig. 1.** Comparison of three different arginase assay methods. The analysis of results using different standard curves with their respective substrate (ornithine and urea) E1 = ornithine estimation by Chinard, E2 = Berthelot's method, E3 = DAM method. All the experiment performed three times independently and values plotted here is mean of the three independent sets.

**Table 1**

Comparison of three different arginase assay methods and their conversion factor. All the experiment performed three times independently and values plotted here is mean of the three independent sets.

Arginase assay methods	Urea ( $\mu\text{mole}/\text{min}$ )	Conversion factor
AAE3	1.11	2.66
AAE2	0.613	1.47
AAE1	0.416	1

\*AAE1 = ornithine estimation by Chinard, AAE2 = Berthelot's method, AAE3 = DAM method.

and all possible experimental conditions suggests a conversion factor (CV) between these methods. For the calculation of the conversion factor, a numerator is divided by a denominator that gives a ratio or conversion factor. Here, the three methods were compared by calculating the value of 1OD of each, for E1  $0.613\mu\text{molornithine}/\text{ml}$ , E2  $1.11\mu\text{mol urea}/\text{ml}$ , and E3  $0.416\mu\text{mol urea}/\text{ml}$ . The one method is taken as a denominator and the other two as the numerator and the ratio comes as a conversion factor from the one method to the other. A conversion factor is used to change the units of measured quantity without changing its value. For example, E3 is taken as a denominator and E1 and E2 were as numerators So, the conversion factor ( $\mu\text{mol urea or ornithine}/\text{ml}$ ) for E3 to E1 is 1.47 whereas for E3 to E2 is 2.66 (Table 1). If we calculate the amount of urea by the DAM method (E3) and want to change it by the chemical method of Berthelot's (E2) multiplication of the Value of E3 into 2.66 will give the comparable value without changing the measured quantity. For example, mice liver tissue gives arginase activity by E3 method  $1013.37 \pm 14.39\mu\text{mole urea}/\text{min}$  for comparing it in E2 method  $1013.37 \pm 14.39\mu\text{mole urea}/\text{min}$  multiplied by 2.66 gives  $1124.84 \pm 15.97\mu\text{mole urea}/\text{min}$ . Another drawback is the lack of suitable control reaction in arginase enzyme assay as endogenous urea and arginine used as substrate could deviate the interpretation also. Sudarshana et al. [12] during an analysis of interference in arginase assay suggested the importance of controls during enzymatic studies, So, we examined different components of the arginase assay system. Table 2 summarizes the amount of urea in different control reactions and the significant amount of urea observed in the arginine blank shown the necessity to take it as one of the control in arginase activity. Rahmatullah and Boyde [13] revisited the reaction conditions and suggested various modifications including removal of the deproteinization step using the DAM-TSC method. They modified the DAM-TSC with  $\text{H}_3\text{PO}_4 + \text{H}_2\text{SO}_4$  with Ferric Chloride. They reported the modified DAM method as the sensitive, simple, and stable method of detection. The ornithine detection of ninhydrin method is also reported to sensitive but ninhydrin is commonly used in the determination of amino acids also. The chemical method of Berthelot's is selectively toward urea hence found an equally appropriate method of urea determination.

#### 4. Conclusion

The determination of the CV between different methods made it feasible to compare different studies as well to get a conclusion that couldn't be done due to different assay conditions. The more impact was taken in this study on the analysis of control reaction since that is an important point in any enzyme assay. Brown and Cohen [8] were used a "perchloric acid-treated system" as a control blank whereas routinely tissue blank is used as a control in the arginase assay but the incorporation of arginine blank could enhance the accuracy of the detection.

#### Author statement

SM and RM planned the experiment and SM had done the

**Table 2**

Summarizes the amount of urea in different control reactions and significant amount of urea observed by in arginine blank shown the necessity to take it as one of control in arginase activity.\*Ml-mice liver tissue extract. All the experiment performed three times independently and values plotted here is mean  $\pm$  SD of the three independent sets.

Arginase assay mixture	Mean $\pm$ SD
Buffer	0
Buffer + $\text{MnCl}_2$	$10.192 \pm 2.59$
Buffer + $\text{MnCl}_2 + \text{Arginine}$	$886.49 \pm 11.19$
Buffer + $\text{MnCl}_2 + \text{Tissue}$	$12.06 \pm 2.88$
Ml tissue	$1013.38 \pm 14.39$
Endogenous Urea	$0.012 \pm 0.001$

experiments. SM and RM wrote the manuscript.

#### Declaration of competing interest

The authors have no conflict of interest.

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