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Modification of Spectroscopic Method for Determination of L-arginine in Different Tissue extracts

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ABSTRACT
A simple technique was established for the specific modification of guanidine groups in different extracts of tissues involving reaction with ninhydrin. The extent of the reaction is simply determined by spectrophotometric analysis. The method compared with HPLC. This modified method made easily determined in micro-quantities of L-arginine, which were operating conditions for the successful determination of arginine were optimized. The results shed the light on reduction of interference substance with and given accuracy as compared with HPLC analytical method with acceptable recovery percent as well as this method given the amount of free type of arginine based on rapid reaction of amino group of L-arginine and compartmentalization was neglected the bound type profile. Finally the procedure was display high reproducibility and sensitivity of different biological tissues extracts, further studies need to explore the pharmacokinetic using of this method for given their applicability.

Keywords: L-arginine, Modification, Spectroscopic Method

1. INTRODUCTION
L-arginine is an essential amino acid particularly for the developing adolescents, which had been documented since the 1889s. L-arginine was involved in numerous metabolic pathways: comprising the synthesis of urea, creatine and agmatine. L-arginine is also the physiological forerunner of nitric oxide, a mediator released by vascular endothelial cells. That accounts for the biological activity of endothelium- derived relaxing factor Metabolic disorders of arginine catabolism can lead to periodical and enduring hyperargininemia, where with the last one, certain cases, but not all of them, are mentally retarded[1,13]. It was necessary to take worthy maintenance with the quantity of nourishment and pharmaceutical preparations (regarding the amount of amino acids). Several analytical methods had been developed for the determination of arginine and the majorities of them were applicable to microgram amounts and have a narrow dynamic range of determination. Among these were spectrophotometry, HPLC with UV detection and turbidimetry. Pulsed amperometry for determination of arginine uses high anodic potential polarization. Ionization mass spectrometry had also been used for the determination of arginine with high sensitivity, but suffers from a more time overriding techniques and expensive instrumentation. Chemiluminescence was an attractive analytical method due to its sensitivity, ease and simplified of operation especially to trace biological substances analysis, but this method had not been widely used in practice. Some studies involved enzymes which made the experiment more expensive and required more care for maintaining the activity of the enzymes[2,16].

Modification method of guanidino-specific reagents manner is needed for the resolve of the precise amount of arginine containing in the different tissues of the animals and plants structure of free form. The guanidino group of arginine (PKa 12.5) had been modified by reaction with several diketones[18]. This reagent exhibited specificity for guanidine groups over amino groups, because the distance between the two carbonyl groups closely matches that of the two non-replaced nitrogen atoms of the guanidino group. However, the reactions involved are unspecific site. The application of this reaction to the modification of the guanidino group of arginine by ninhydrin had been explored and the specificity of their action determined with amino acids. The reaction had been used to examine the exposure of the arginine contain in the liquid extracted and in mixture or combined drugs[3].

In continuation of arginine studies a simple technique was developed for the specific reversible modification of guanidine groups in proteins involving reaction with ninhydrin. The extent of the reaction is easily determined non-destructively by spectrophotometric analysis[3,17]. The reagent can also be used for the titration of thiol groups in proteins which were prospective determination of L-arginine in different biological fluids and pharmaceutical preparations.

2. MATERIAL AND METHODS
In order to determine the lowest possible determine able concentration of arginine, the conditions needed to be optimized. Therefore, the dependence of the ninhydrin reactions on the concentration of each of the reactants was determined as follows:

2.1 *Reagents*
Ethyl alcohol (80%), Chloroform, deionized water, sodium dihydrogen phosphate, disodium hydrogen phosphate, ninhydrin (Fluka,Co).

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2.2 *Apparatus*
UV-Visible spectrophotometer (Apel, Co.), PH meter (Hanna).

2.3 *Extraction and sampling of different biological fluids*
There were different methods for extraction and sampling of several biological fluids, one of these methods is as follows:- The reaction was carried out in the following way, In since the samples are protein complex and therefore had small quantities of amino acids apart from the large quantities of other substances such as vitamins and proteins in the form of gluconates, and considering their given proportion in the sample, it was necessary to combine several methods for isolation and determination of arginine. Firstly, amino acids were separated using 80% ethylalcohol and the proteins were deposited by chloroform; next, the base amino acids (histidine, lysine, arginine) were separated; finally, arginine was separated from lysine andhistidine.0.5 g of the sample was placed into an Erlenmeyer flask and 4 mL of deionized water was added together with16 mL of absolute alcohol. The contents were shaken for half an hour and moved into a 100 mL separation funnel where 60 mL of chloroform was added and all was well shaken the upper water[4].

The base amino acids were carried out according to[14] formulated a method for the separation of base amino acids: arginine, histidine and lysine. A warm concentrated solution of Ag_2SO_4 in a small surplus was added to the water solution of amino acids. Arginine and histidine were deposited out of the solution by adding Ba $(OH)_2$, while lysine remained in the solution. These two amino acids were then separated on the basis of the different properties of their respective silver salts at pH= 7: silver salt of histidine deposits, whereas arginine remains in the solution. Silver ions were removed by adding (0.1) mol L^{-1} $Na_2S_2O_3$ solution which binds silver ions into a stable complex. Employing multiple chloroform extractions, complex of Ag^+ with $S_2O_3^{-2}$ was extracted from the water solution.

2.4 *Procedure*
Several steps were done to determine concentration of L arginine according to[5] with modification [19]

2.4.1 *Preparation of phosphate buffer solution*
The phosphate buffer solution at PH 9.0 was prepared by mixing appropriate volume of 0.2M disodium hydrogen phosphate (50) ml and 0.2 M sodium dihydrogen phosphate (0.35) ml.

2.4.2 *Preparation of ninhydrin solution*
Ninhydrin reagent (2%) was prepared by dissolving 2.0g of ninhydrin in phosphate buffer, PH 9.0 (100) ml.

2.4.3 *Preparation of L-arginine standard solution*
The stock solution of L-arginine at concentration of 3mg/ml was prepared by dissolving 30 mg of L-arginine in 10.0 of phosphate buffer, PH 9.0. Then, 1.0 ml of solution was transferred into 25 ml volumetric flask and diluted to the mark set point with the same buffer (0.12) mg/ml.

2.4.4 *Preparation of calibration solution of L-arginine*
Serial dilutions of L-arginine were prepared by transferring (0.3, 0.35, 0.4, 0.45, 0.50, 0.55, 0.60) mg/ml,of standard solution of L-arginine(0.12) mg/ml separately in to series of the glassed test tubes, then the test tubes were extended and diluted to (2) ml with phosphate buffer PH 9.0and plus 1.0ml aliquot of ninhydrin reagent was added and finally the end concentration were became (0.012, 0.014, 0.016, 0.018, 0.020, 0.022 and 0.024). The resulting mixtures were heated in water bath at 80C° for 15min. After cooling, they were subjected to spectrophotometric analysis at λ=404nm against the mixture of 2 ml of phosphate buffer, PH 9.0 and 1ml of ninhydrin reagent as the blank and the absorbance of these mixtures were recorded. A calibration graph was obtained by regression of the standard L-arginine concentration against absorbance of certain concentration. This calibration graph was used to calculate the concentration of L-arginine in unknown samples.

2.5 *Determination of L-arginine in Pharmaceutical Preparations and biological fluids*
Phosphate buffer solution (2) ml of PH 9.0 and 1ml of ninhydrin solution were transferred separately into series of the glassed test tube. 0.1-0.3 ml of the different biological extracts from plants and animals fluids was added to the contents of each test tube. The resulting mixtures were heated in water bath at 80 C° for 15 min. After cooling they were subjected to spectrophotometric analysis at λ=404 nm against the mixture of 2 ml of phosphate buffer, PH 9.0 and (1) ml of ninhydrin reagent as the blank and the absorbance of these mixtures were recorded.

3. RESULTS

3.1 *Interfering Substances*
After reaction with ninhydrin, the following substances did not yield significant increases in optical densities at 515nm when present in molar concentrations 100 times as great as those of L-arginine: leucine, isoleucine, serine, valine,

threonine, glutamic acid, glycine, alanine, and amino valeric acid, urea, creatine, creatinine, glycocyamine, glucosamine, ammonium chloride, pyrrolidone carboxylic acid. The following substances did not yield significant increases in optical densities at 515 nm when present in molar concentrations 10 times as great. As those of L-arginine: glutamine, histidine, cystine, methionine, tryptophan, tyrosine, hydroxyproline, aspartic acid, asparagine, phenylalanine, 1,4-diaminobutane, 1,5-diaminopentane. The following substances yield significant increases in optical densities at 515 nm when present in molar concentrations 10 times as great as those of L-arginine: citrulline, cysteine, lysine, and hydroxyl lysine. The interference produced by arginine is probably not the result of production of ornithine during the reaction with ninhydrin: the absorption coefficient varies from sample to sample and there is no increase in the optical densities of the solutions when these are heated for longer than the specified 60 minutes. It appears that small and variable amounts of a contaminant, possibly preformed ornithine may be present in the several samples of arginine examined. As is evident from cysteine produces a characteristic color; this color is not produced with cystine. The cysteine reaction product has not been identified. The interference by citrulline may be due to hydrolysis of this amino acid to yield proline. Tryptophan gives a canary yellow color, with a peak at 320 nm. Agreement with Bouguer-Beer Law-A linear relationship is obtained between concentrations of amino acid.

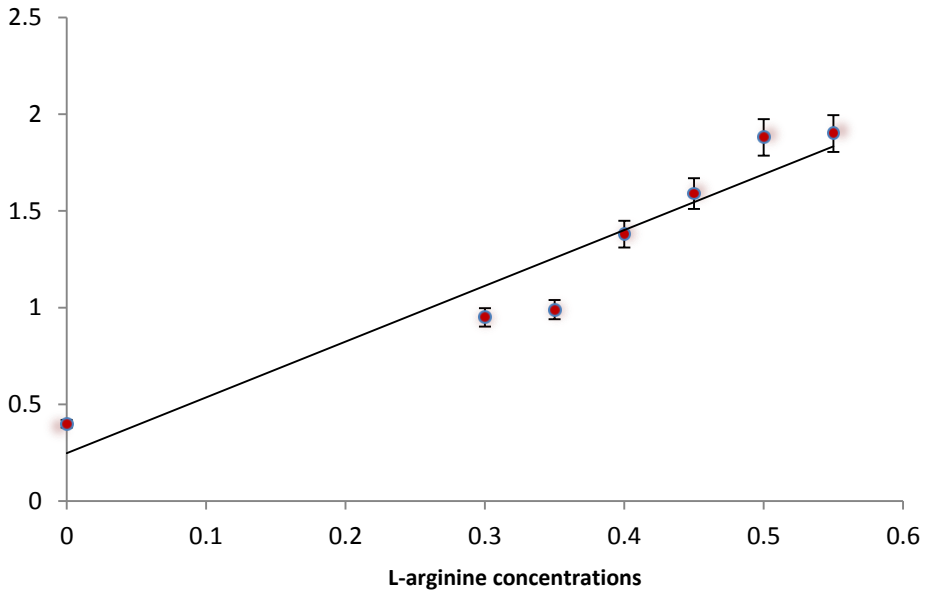

Fig-1: Standard calibration curve of L-arginine concentration vs. absorbance

4. DISCUSSION

The applicability and limitations of the technique were obvious from the results. The technique was best suited for the identification and estimation of the L-arginine indicated above in those instances in which interfering substances had been removed, for example by extracted plant and animal samples as well as direct biological fluids forms (6), or in which was interfering substances were known to be absent. The procedure had already been applied by several investigators. It must be emphasized that the possible interference of peptides and particularly of polypeptides had not been investigated. It is tentatively suggested that the L-arginine undergoes oxidation, deamination and decarboxylation and that the resultant aldehyde group condenses with the terminal amino group with formation of a ring. This cyclic product then couples with 2 molecules of ninhydrin to form the colored product[7,14]. The appearance of a yellow color in the reaction of L-arginine, not apparent in the reaction of amino acids, suggests that a side reaction occurs.

This could account for the fact that less color is produced by reaction of L-arginine than by reaction of other amino acid with ninhydrin[8,15].

Ninhydrin solutions in distilled water gave a U.V. spectrum similar to that obtained by[5], display a chief peak at (404) nm, and given concentration (0.012, 0.014, 0.016, 0.018, 0.020, 0.022 and 0.024) respectively, all of which varied slightly with respect to their found amount of L-arginine. When the ninhydrin solution was allowed to react with solutions of various amino acids or proteins at 37C°, the (404) nm absorption gradually disappeared, and this decrease was used as a measure of the extent of reaction (Table 1).The reaction between arginine and ninhydrin took place in two stages, an initial rapid reaction to an equilibrium state followed by a very slow reaction(1000-fold slower) of similar velocity (11 pmol of ninhydrin reacted/min per mol of L-arginine) to that between glycine and ninhydrin (151nmol of ninhydrin reacted/min per mol of glycine) under the same conditions of temperature (37°C) and pH (9.1).During the first rapid reaction there was no reduction in the 254nm absorption peak, display a similarity to the

reaction between ninhydrin and guanidine[3], but this peak lessened during the later actual slow reaction phase in a ninhydrin was allowed to react with an amino acid or protein in a 0.1 M-NaH_2PO_4/Na_2HPO_4 buffer at 37C°, and the

Table-1: Determination of L-arginine concentration in different tissue and fluids extracts in both spectrophotometric and HPLC methods

Methods of Measurements (extracts)	Mucus of cow vagina extract	Cow saliva extracts	Cow Semen fluid extract	Nut extracts extract	L-arginine pharmaceutics preparation extract
Spectrophotometric measurement of L-arginine mg/ml	0.0048±0.00096 a	0.0099±0.00010 a	0.0097±0.00040 a	0.0314±0.0092 a	0.0201±0.0036 a
Spectrophotometric method recovery %	90.31±5.701	88.83±6.593	98.20±1.845	96.14±3.817	99.21±1.872
HPLC measurement of L-arginine mg/ml	0.0049±0.00038 a	0.0108±0.00617 a	0.0099±0.00114 a	0.0333±0.0075 a	0.025±0.001 b

Mean ± SE, n=8, Letters denoted to significant at P<0.05

extent of the reaction was determined by the loss in absorbance at 232nm. Similar manner to that happened in the reaction among ninhydrin and serine, glycine, histidine, lysine and cysteine[4].

This suggested that the initial rapid reaction was due to an exact reaction of ninhydrin with the guanidino group and that the later slow reaction was due to the non-specific reaction of ninhydrin with the free amino groups of these amino acids. Ninhydrin reacts with arginine very rapidly compared with other amino acids, except cysteine[8].

The initial rate of this reaction was found to be proportional to the concentration of ninhydrin and of L-arginine and to the OH- ion concentration, (Table 1).

This agrees with[22] in that the free thiol group[21] of papain is suppressed deep in the molecule, but the rate indicates that most of the arginine residues[21,11] were also sterically delayed at least to the extent that reaction with ninhydrin was concerned. The reaction between cysteine thiol groups and ninhydrin was rapid and energies essentially to completion. It can be used for the adjustment and titration of these groups, when they are not steric-allyl protected or for the resolve of the degree of steric protection. The ninhydrin- arginine reaction could be used for the titration of steric-allyl unprotected arginine group sin proteins and other contaminate for the change of proteins for investigation into their structure/activity locus. After reaction at alkaline pH; the protein can be kept for some time at acid pH for study free from excess of reagents[12]. This reaction can usefully be applied to standardize and estimation of reactive amount of unbound L-arginine and a kinetic studies on the allosteric and active sites of enzymes, with low interference due to side reactions. These applications, involving a relatively cheap and readily available reagent, are simplified by the ease with which the rate and degree of reaction can be determined non-destructively by U.V. spectrophotometry[9,10].

This work of measurement of L-arginine provides further evidence of L-arginine compartmentalization. They demonstrated that a simple exists between spectrophotometric and compared with HPLC methods in different that associated with a major free L-arginine contents protein in biological fluids extracts and pharmaceutics compound[2]. These data suggested that L-arginine is sequestered from the intracellular L-arginine supply by being located within compartmentalization and dependent upon the direct transfer of L-arginine into this extracellular compartment by the system y^+ transporter. If the transporter function is decreased as may occur with oxidative injury, the L-arginine supply could immediately become limiting and be the basis of determination of variation. L-arginine intakes and endogenous may affect the delivery of arginine to the external excretion and, consequently, to the inner compartmentalization. One of the limitations in this study is we did not measure bound amino acid levels in the cells and had no information about the ambient amount of L-arginine storage from protein intake.

As well as there were no significant differences occur between two methods in their values give an impression could use to study of L-arginine pharmacokinetic[20].

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