VALIDATION OF HPLC METHOD FOR DETERMINATION OF L – ARGININE IN TONOTYL® SOLUTION

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ABSTRACT
An isocratic HPLC method with UV – detection for quality control of L – Arginine in Tonotyl® solution is validated in respect of analytical parameters selectivity, accuracy, precision and linearity. For model mixtures with added quantity of 80 mg, 100 mg and 120 mg L – Arginine, precision is estimated by standard deviation (SD) and relative standard deviation (RSD) (%): SD = 1.76, RSD = 2.20 % (80 mg); SD = 2.50, RSD = 2.50 % (100 mg); SD = 1.61, RSD = 1.32 % (120 mg). Accuracy is presented by the degree of recovery R (%) ± RSD (%): 99.66 % ± 0.71 (80 mg); 99.99 % ± 2.20 (100 mg); 100.37 % ± 0.24 (120 mg). The obtained regression equation for L – Arginine is: y = 2.10 ± 0.24 x – 98532, R² = 0.996. The applied HPLC analytical procedure can be used for quality control of food supplements, containing 100 mg L – Arginine, with great precision and accuracy in appointed linear interval.

Keywords: L – Arginine, HPLC, Accuracy, Precision, Linearity.

INTRODUCTION
Amino acids are of great importance for human health and especially for football players [1] due to their different roles. L – Tyrosine is useful in following conditions: depression, Parkinson’s disease, phenylketonuria and vitiligo 2. L – Lysine prevents of herpes and lowers the severity of osteoporosis by increasing the absorption and reducing the excretion of calcium 3. L – Leucine is involved in protein synthesis in the skeletal muscles 4. L – Arginine is an amino acid, involved in numerous areas of human biochemistry, including: 1) changing into powerful neurotransmitter nitric oxide, which modulates it’s biological effect by activating the soluble isoform of guanylyl cyclase and increasing the synthesis of nitric oxide, which mediates it’s biological effects by activating the cyclic GMP – dependent protein kinase (PKG), causing by this way: a) smooth muscle relaxation and blood vessel dilatation; b) decreasing of blood pressure and platelet aggregation; c) improving of blood flow in the arteries of the heart [5]; vascular function [6] and muscle metabolism; 2) reversing of endothelial dysfunction in hypertensive cardiac transplant recipients, hypercholesterolemic patients and in cigarette smokers; 3) ammonia detoxification; 4) regulation of growth hormone production; 5) enhancement of the spermatogenesis; 6) stimulation of: a) insulin secretion from pancreas, b) synthesis of the pituitary hormone vasopressin [7]; c) immune system, by increasing the output of T – lymphocytes from the thymus gland [8]; 7) prevention of wasting in people with critical illnesses [7]; 8) improvement of survival in gut – derived sepsis and peritonitis by modulating bacterial clearance 5, 9. Arginine vasopressin is nonapeptide, which regulates hypothalamus – pituitary adrenal system by enhancing the effects of corticosterone releasing factor on adrenocorticotropic hormone release 10. L – Arginine is used as a component of media for isolation of actinobacteria 11.

For the determination of L – Arginine are developed the following methods: 1) second derivative UV – spectrophotometry (in injections in combination with Cephradine) [12]; 2) VIS – spectrophotometry by measurement of the absorbance of derivative product of reaction between L – Arginine and thymol reagent at λmax = 440 nm (in protein hydrolyzate) [13]; 3) indirect determination by graphite furnace atomic absorption spectrometry, after preconcentration on a nafion chemically modified tungsten coil [14]; 4) fluorimetry, after reaction with 2,3 – naphthalenedicarbaldehyde at λexcitation = 462 nm, λemission = 520 nm (in serum) [15]; 5) isocratic RP HPLC with: a) UV – detection with lbpurofen [16]; b) fluorescence detection by derivatization with naphthalene – 2,3 – dicarboxaldehyde (in human plasma) [17]; c) electrospay ionisation (ESI) in the positive mode (in human plasma) [18]; d) gradient RP HPLC with: a) UV – detection at λ = 250 nm (in human plasma) [19]; b) chemiluminescence detection after the Sakaguchi reaction [20]; c) MS coupling with an atmospheric pressure chemical ionization (APCI) (in human urine) [21]; 7) Hydrophilic – Interaction Liquid Chromatography – MS (ESI) [22]; 8) High – Resolution Capillary Electrophoresis (combination with Raghagitaz in tablet) 2.3.

The aim of current study to validate in respect of analytical parameters: precision, accuracy and linearity an isocratic HPLC method with UV – detection for quality control of L – Arginine in Tonotyl® solution and in food supplements, containing 100 mg L – Arginine.

MATERIALS
I) Reference standard (RS): L – Arginine. II) Reagents with analytical grade quality: 20 mM ammonium acetate, distilled water.

METHODS
HPLC
Chromatographic system
Liquid chromatograph Shimadzu (Japan) (LC – 10 Advp), equipped with: analytical column RP – 18 ODS (250 mm/4.6 mm id. / 5 µm), column oven (CTO – 10 Asvp Shimadzu); isocratic pump (LC – 10 Asvp); UV – detection for quality control of L – Arginine in Tonotyl® solution and in food supplements, containing 100 mg L – Arginine.

Chromatographic conditions
Mobile phase (MPH): 20 mM ammonium acetate; flow rate: 1.0 ml/min; column temperature: 40 °C; UV – detection at λ = 254 nm; volume for injection – 20 µl. Before using MPH was filtered through membrane filter with pore size 0.45 µm.

Accuracy and precision (repeatability)
1) Preparation of model mixtures with L – Arginine
Three (3) equal homogenous model mixtures (MM) were prepared from all respective supplements (magnesium stearate, microcrystalline cellulose), by adding of RS L – Arginine, equivalent to 80 % (80 mg), 100 % (100 mg), 120 % (120 mg) of theoretical concentration. An average weight of MM were: 0.38 g (A100), 0.42 g (A120), 0.42 g (A120). From each MM were prepared 3 samples, by dissolving in volumetric flask an accurately weighed quantity, containing RS L – Arginine: 80 mg, 100 mg, 120 mg to 100.0 ml with MPH.

2) Preparation of standard solutions from RS L – Arginine
An accurately weighed quantity: 80 mg, 100 mg, 120 mg of RS L – Arginine was dissolved in MPH to 100.0 ml to obtain solutions with

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Research Article
The "placebo" solution with all labeled in Tonotyl® solution supplements (magnesium stearate, microcrystalline cellulose), without the active ingredients were prepared at the same manner, like reference standard of L-Arginine. The selectivity of the applied HPLC method was proved by the fact, that on chromatogram with "placebo" solution didn’t exist peaks with tS, correspond to retention time: tS = 2.9 min., obtained by reference standard L-Arginine and by L-Arginine in Tonotyl® solution.

Analytical parameters accuracy, precision and linearity for the validation procedure for HPLC method for determination of L-Arginine were recorded.

RESULTS AND DISCUSSION

A) Validation of HPLC method for determination of L-Arginine for analytical parameters selectivity, accuracy, precision (repeatability) and linearity.

Selectivity

The "placebo" solution with all labeled in Tonotyl® solution was dissolved in MPh to 100.0 ml to obtain solutions with concentration correspondingly: 8.10⁻³ g/ml; 1.10⁻³ g/ml; 1.2.10⁻³ g/ml L-Arginine. All solutions were filtered through membrane filter with pore size 0.45 µm. The written HPLC method was applied and chromatograms were recorded.

Table 1: Added content of RS L-Arginine in MM and weighed quantity of MM

<table>
<thead>
<tr>
<th></th>
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<td>0.4056</td>
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<td>3.</td>
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Table 2: High of peak (H) for model mixtures of RS L-Arginine and Chauvenet’s criterion for H

<table>
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<tr>
<th>N:</th>
<th>H A₈₀</th>
<th>U H A₈₀</th>
<th>H A₁₀₀</th>
<th>U H A₁₀₀</th>
<th>H A₁₂₀</th>
<th>U H A₁₂₀</th>
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<td>2.</td>
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<td>0.38</td>
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<td>57690</td>
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<td></td>
<td>56207</td>
<td></td>
<td>90790</td>
<td></td>
<td>135067</td>
<td></td>
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</table>

The content of L-Arginine is obtained by method of reference standard. On Table 3, are indicated: N – number of the individual measurements (1 ÷ 3); [A] – obtained quantity of L-Arginine in model mixtures: [A₈₀], [A₁₀₀], [A₁₂₀]; U [A] – Chauvenet’s criterion for [A]; U[A₈₀], U[A₁₀₀], U[A₁₂₀]; R (%) – degree of recovery: R[A₈₀], R[A₁₀₀], R[A₁₂₀]; X – arithmetical mean; SD – standard deviation; RSD (%) – relative standard deviation;  S X – mean quadratic error; P – confidence possibility (%); t – coefficient of Student; tS X ± tS X ÷ X ± tS X – confidence interval (CI); E – relative error.

Table 3: Accuracy and precision for L-Arginine – estimation by method of calibration curve

<table>
<thead>
<tr>
<th>N:</th>
<th>[A₈₀] [mg]</th>
<th>R[A₈₀] [%]</th>
<th>U[A₈₀] [g]</th>
<th>[A₁₀₀] [mg]</th>
<th>R[A₁₀₀] [%]</th>
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<td>120.12</td>
<td>100.10</td>
<td>1.14</td>
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<tr>
<td>2.</td>
<td>79.29</td>
<td>99.86</td>
<td>0.31</td>
<td>99.25</td>
<td>98.95</td>
<td>0.38</td>
<td>122.58</td>
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<td>3.</td>
<td>81.80</td>
<td>100.25</td>
<td>1.12</td>
<td>103.03</td>
<td>102.52</td>
<td>1.14</td>
<td>123.16</td>
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<td>0.75</td>
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Table 4: Accuracy and precision for L-Arginine – estimation by method of calibration curve

<table>
<thead>
<tr>
<th>N:</th>
<th>[A₈₀] [mg]</th>
<th>R[A₈₀] [%]</th>
<th>U[A₈₀] [g]</th>
<th>[A₁₀₀] [mg]</th>
<th>R[A₁₀₀] [%]</th>
<th>U[A₁₀₀] [g]</th>
<th>[A₁₂₀] [mg]</th>
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<td>1.14</td>
<td>123.16</td>
<td>100.54</td>
<td>0.75</td>
</tr>
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</table>
For all of the obtained by the applied HPLC method data for high of peak and content of L-Arginine in each sample is necessary to estimate U, because when U for one value is higher than the relevant standard criterion (USL), the result must be removed as unexpected.

The relations: UH < 1.68 (Table 2.) and UC < 1.68 (Table 4.) show, that all experimental results for UH and UC are lower, than standard requirement: U max = 1.68 (n = 3), and it isn’t necessary to remove data for H and C.

For the assessment of accuracy and precision is calculated sample standard deviation (SD), by the applying of the Bessel’s correction, in which the denominator N − 1 (degrees of freedom) is used instead of N and in this case (S^2) is an unbiased estimator for (SD)^2.

Analytical parameter accuracy is presented by the degree of recovery R (%) and RSD (%). All results for R suit relevant confidence interval: [A100%]: 98.46 ± 100.86 (RSD = 0.71); [A120%]: 96.28 ± 103.70 (RSD = 2.20); [A150%]: 99.96 ± 100.78 (RSD = 0.24).

For the estimation of an analytical parameter precision (repeatability) is used the uncertainty of the result, which is determined by SD, RSD and \( \bar{X} \) ± t. S\( \bar{X} \). From the assessment of precision [25], it is obvious, that the content of L-Arginine in model mixtures correspond to the relevant confidence interval: [A100%]: 76.85 ± 82.81 (SD = 1.76, RSD = 2.20); [A120%]: 95.96 ± 104.42 (SD = 2.50, RSD = 2.50); [A150%]: 119.23 ± 124.67 (SD = 1.61, RSD = 1.32).

### Linearity

For the investigation of analytical parameter linearity the prepared 3 solutions with decreasing concentration of reference standard L-Arginine are analyzed separately by the written HPLC method. The proportional accordance between the peak high (H) and concentration (C) in g/ml is found and the results are shown on Table 4.

#### Table 4: Results for analytical parameter linearity for L-Arginine.

<table>
<thead>
<tr>
<th>N</th>
<th>Concentration [g/ml]</th>
<th>High of peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.10^-2</td>
<td>551.12</td>
</tr>
<tr>
<td>2</td>
<td>1.10^-3</td>
<td>892.46</td>
</tr>
<tr>
<td>3</td>
<td>1.210^-3</td>
<td>1312.46</td>
</tr>
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</table>

The results are put into linearity regression analysis and the coefficient of regression (R) is calculated. The obtained regression equation, showing the proportional accordance H = f (C) is: \( y = 21.8 \times 10^{-4} - 9.8532 \). R^2 = 0.996. The calibration curve, presented the linearity of L-Arginine is illustrated on Fig. 1.

**CONCLUSION**

All data for \( g [\%] \) ± RSD (%) suit respective confidence intervals: A100%: 99.66 ± 0.71; A120%: 99.99 ± 2.20; A150%: 100.37 ± 0.24.

The obtained quantities of L-Arginine ( .. ± SD) in model mixtures are correspondingly: A100%: 79.83 ± 1.76; A120%: 100.19 ± 2.50; A150%: 121.95 ± 1.61.

The validated HPLC method is appropriate for determination of L-Arginine with great accuracy and precision in Tonotyl® solution and in other food supplements with the same content (100 mg) of L-Arginine.

**REFERENCES**

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