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Identification and determination of L–Glutamic acid and L–Arginine by HPLC in food additives

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Abstract

New analytical method for quality control of L – Glutamic acid and L – Arginine in food additives by terms of HPLC is developed and validated in respect of analytical parameters: selectivity, precision, accuracy, linearity, limit of detection = 4.0 µg/ml, limit of quantitation = 40.0 µg/ml. Precision is estimated by SD and RSD (%): SD = 0.02, RSD = 4.76 % (L – Glutamic acid in Tonotyl ® solution); SD = 0.05, RSD = 4.85 % (L – Arginine in Sargenor ® amp.). Accuracy for L – Glutamic acid is represented by the degree of recovery – R (%): 99.19 % ÷ 102.08 % and RSD (%): 1.90 % ÷ 5.69 %.

Keywords: L – Glutamic acid, L – Arginine, HPLC, food additives.

Introduction

L – Glutamic acid is non – essential proteinogenic amino acid, important for the metabolism of sugars and fats and for synthesis of Glutamine, GABA, Glutatione and proteins. L – Glutamic acid is excitatory neurotransmitter, acts as fuel for the brain, improves mental capacities and cognitive functions like learning and memory, detoxifies the brain and body of ammonia (Balch 2006), helps in the transportation of potassium across the blood – brain barrier (Smith, 2000) by a high affinity transport system (Shigeri et al. 2004). L – Glutamic acid shows promise in the treatment of neurological diseases, mental retardation, personality disorders, epilepsy, Parkinson's, ulcers, hypoglycemic come, benign prostatic hyperplasia, muscular dystrophy and has antiishemic effect in people with stable angina pectoris (Balch 2006, Braverman 1997). In cases as brain injury, disease or damage the blood – brain barrier, L – Glutamic acid accumulates outside brain cells and causes cell death (excitotoxicity) by damaging of mitochondria from excessively high intracellular Ca^{2+} and L – Glutamic acid/Ca^{2+} – mediated promotion of transcription factors for pro – apoptotic genes (Balch, 2006). L – Arginine is an essential, proteinogenic amino acid (Böger and Bode – Böger 2001, Böger 2007) and is involved in metabolic pathways, such as protein degradation, synthesis of creatine, citrulline, L – ornithine, L – glutamate, agmatine, proline, polyamines, urea (Grillo and Colombatto, 2004).

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L–Arginine stimulates synthesis of nitric oxide (Napoli et al. 2006) – mediator in nonadrenergic, noncholinergic neurotransmission; important for neuroprotection, learning and memory, synaptic plasticity and dilatation of small blood vessels in brain (Böger 2007, Braverman 1997); vasodilatator (Li et al. 2006); antiaterogenic factor (Napoli et al. 2006); mediator of host defense in immune system (Böger 2007); potent inhibitor of osteoclastic bone resorption (Visser and Hoekman 1994). By raisening of polyamine levels, may prove helpful in treating Alzheimer's (Böger and Bode – Böger 2001, Braverman 1997). L – Arginine improves vascular function (Böger and Ron 2005) and exercise capacity in patients with congestive heart failure (Bednarz et al. 2004); enhances vasodilation (Böger and Ron 2005) in patients with coronary artery disease (Blum et al. 2000); decreases blood pressure in patients with precapillary pulmonary hypertension (Nagaya et al. 2001, West et al. 2005); reduces endothelial monocyte adhesion (Bode – Böger et al. 2003) and platelet aggregation (Böger and Bode – Böger 2001), but should not be recommended orally, following acute myocardial infarction (Abumrad and Barbul 2006, Bednarz et al. 2005). This amino acid promotes lymphocyte production (Böger and Bode – Böger 2001, Braverman 1997) and by enhancing immune function (Balch 2006), retards tumor growth (Böger and Bode – Böger 2001, Braverman 1997). In combination with proanthocyanidins (Stanislavov and Nikolova 2003) or yohimbine (Lebret et al. 2002), improves erectile dysfunction (Balch 2006). By neutralizing ammonia, helps for liver detoxification, and for the treatment of liver injury, hepatic cirrhosis and fatty liver degeneration (Balch 2006, Böger and Bode – Böger 2001). L – Arginine is a component of collagen and plays an important role in cell division and in reducing healing time of injuries (Balch 2006, Braverman 1997). L– Arginine is a component of hormone vasopressin; stimulates the pancreas for insulin production (Böger and Bode – Böger 2001); releases human growth hormone (HGH), by blocking the secretion of the HGH – inhibitor somatostatin (Balch 2006, Visser and Hoekman 1994). HGH increases muscle mass and burning fat (Balch 2006) and reversing many of the effects of aging (Böger and Bode – Böger 2001, Braverman 1997). For the determination of L – Arginine are presented the following methods: spectrophotometry (Yamasaki et al. 1981); fluorimetry (Smitha and MacQuarrie 1978); chemiluminescence (Hi et al. 1990). HPLC method with precolumn derivatization with of o – phthalaldehyde (OPA), followed by reverse – phase separation and fluorescent detection is described for the simultaneously determination of L – Arginine and L – Glutamic acid (Gardner and Miller 1980) and for analysis of L – Arginine in human plasma (Teerlinka et al. 2002).

The aim of this study is to develop and to validate the HPLC analytical method, with greater selectivity, accuracy and repeatability, which can be applied for quality control of food additives, containing L – Glutamic acid and L – Arginine, like Tonotyl ® solution, Sarge – nor ® amp., Arginine Plus ®.

Materials and Methods

I. Materials

1. Drug products:

A) Tonotyl ®

Pulvis:	Solution:
L – Phosphotreonine – 0.01 g	L – Phosphoserine – 0.04 g
L – Glutamic acid – 0.06 g	L – Arginine – 0.1 g
Vitamin B_{12} – 0.004 g	

B) Sargenor ® amp.: Arginine aspartate 1.0 g/5 ml, saccharose, destilled water, methylhydro – xybenzoate, propylhydroxybenzoate.

2. Reference substances: L – Glutamic acid CRS, L – Arginine CRS

3. Reagents: methanol Lichrosolv ® for chromatography (Merck; Germany); bidestilled water, suit to requirements of Ph. Eur. V; ammonium acetate with chemical purity.

II. HPLC method

1) Chromatographic system: Liquid chromatograph Shimadzu (Japan) (LC – 10 Advp), equipped with: column Spherisorb RP – 18 ODS (250 mm/4.6 mm), column oven (CTO – 10 Asvp); isocratic pump (LC – 10 A); UV – VIS – detector at fixed wavelengths (SPD – 10 Avvp); 20 µl injector loop.

2) Chromatographic conditions: flow rate – 1.0 ml/min; column temperature – 25 ^{o}C; 210 nm analytical wavelength; mobile phases: methanol:destilled water – 50:50 v/v and 20 mM solution of ammomium acetate. Before using, the obtained solvent mixtures (mobile phases) are mixed and filtered throught membrane filter with pore size 0.45 µm.

3) Standard preparation of L – Glutamic acid. An accurately weighted quantity (0.01 g) of reference substance L – Glutamic acid is dissolved in 20 mM solution of ammonium acetate in volumetric flask of 10.0 ml.

4) Standard preparation of L – Arginine. An accurately weighted quantity (0.01 g) of reference substance

L – Arginine is dissolved in 20 mM solution of ammonium acetate in volumetric flask of 10.0 ml.

5) Standard preparation of L – Glutamic acid and L – Arginine. An accurately weighted quantities (0.01 g) of reference substances L – Glutamic acid L – Arginine are dissolved in 20 mM solution of ammonium acetate in volumetric flask of 10.0 ml.

6) Sample preparation of Tonotyl ® solution.

1.0 ml of fl. Tonotyl ® solution is diluted with 20 mM solution of ammonium acetate in volumetric flask of 10.0 ml.

7) Sample preparation of Sargenor ® amp.

The quantity of one amp. Sargenor ® (1 g/5 ml) is diluted with destilled water in volumetric flask of 100.0 ml. 1.0 ml of the obtained solution is diluted with 20 mM solution of ammonium acetate in volumetric flask of 10.0 ml.

Results

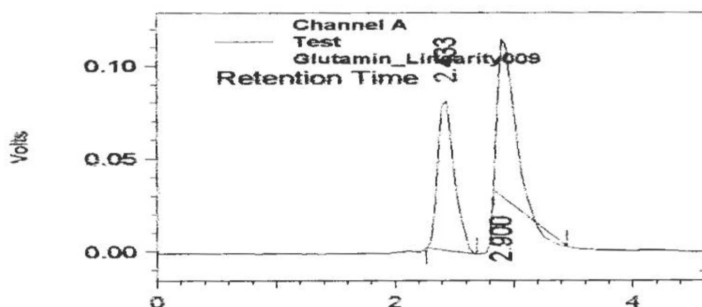
By the applied HPLC method the following results for the retention times of the examined aminoacids (substances and in Tonotyl ® solution) are obtained: in 20 mM ammonium acetate – $t_R = 2.433$ (L – Glutamic acid reference substance – Figure 1.), $t_R = 2.4$ (L – Glutamic acid in Tonotyl ® solution Figure 2), $t_R = 2.9$ (L – Arginine reference substance – Figure 1 and in Tonotyl ® solution – Figure 2); in methanol:destilled water (1:1) both for substances and in Tonotyl ® solution) – $t_R = 5.333$ (L – Glutamic acid – Figure 3.), $t_R = 2.4$ (L – Arginine);

On Figure 1 is reported chromatogram of reference substances L – Glutamic acid and L – Arginine in mobile phase 20 mM solution of ammonium acetate.

On Figure 2 is shown chromatogram of L – Glutamic acid and L – Arginine in Tonotyl ® solution in mobile phase 20 mM solution of ammonium acetate.

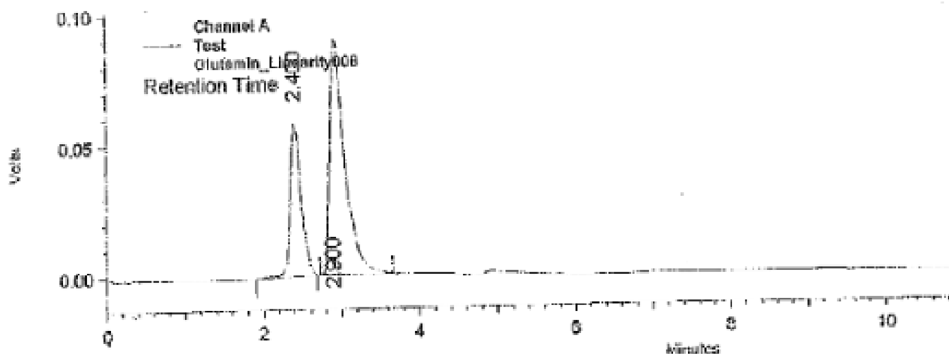
Chromatogram of L – Glutamic acid in Tonotyl ® solutio in mobile phase methanol:destilled water (1:1) is presented on Figure 3.

Figure 1. Chromatogram of reference substances L – Glutamic acid in L – Arginine in mobile phase 20 mM solution of ammonium acetate.



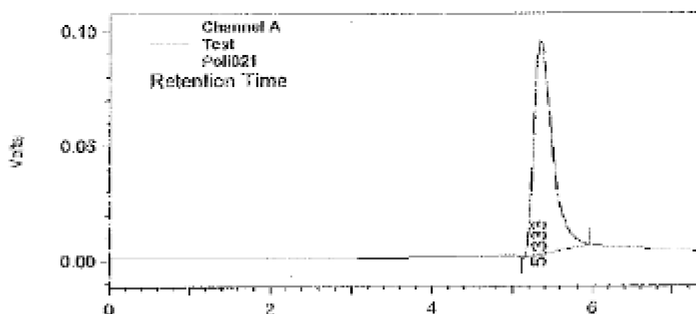
Peak	Retention Time	Area	Are%	Height	Height%
1	2.433	774322	49.224	79916	48.628
2	2.900	798729	50.776	84424	51.372
	Total	1573051	100.000	164.340	100.000

Figure 2. Chromatogram of L – Glutamic acid and L – Arginine in Tonotyl ® solutio in mobile phase 20 mM solution of ammonium acetate.



Peak	Retention Time	Area	Are%	Height	Height%
1	2.433	582054	33.035	58697	39.675
2	2.900	1179878	66.965	89246	60.325

Figure 3. Chromatogram of L – Glutamic acid in Tonotyl ® solutio in mobile phase methanol:destilled water (1:1).



Peak	Retention Time	Area	Are%	Height	Height%
1	5.333	1507625	62.431	92.781	88.624

Validation of HPLC method for analysis of L – Glutamic acid and L – Arginine

1. Precision

In Table 1 are presented the results for the content of: 1) L – Glutamic acid (C_{L-Glu}) (mg) in Tonotyl ® solution and the values of the respective Shovene's criterion for all of the obtained data of L – Glutamic acid ($U C_{L-Glu}$); 2) L – Arginine (C_{L-Arg}) (mg) in Sargenor ® amp. and

the values of the respective Shovene's criterion for all of the obtained data of L – Arginine (U C_{L-Arg}). In Table 1 are indicated: N – number of the individual measurements (1 ÷ 6); $\overline{X}$ – arithmetical mean; SD – standard deviation; RSD – relative standard deviation; S $\overline{X}$ – standard error of arithmetical mean; t – coefficient of Student; P – confidence possibility (%); $\overline{X}$ ÷ t.S $\overline{X}$ – confidence interval; E – relative error.

Table 1. Obtained by HPLC quantity of L – Glutamic acid (C_{L-Glu}) in food additive Tonotyl ® solution and of L – Arginine (C_{L-Arg}) in food additive Sargenor ® amp. and data for respective Shovene 's criterion (U C_{L-Glu}; U C_{L-Arg}).

N:	C_{L-Glu} [mg]	U C_{L-Glu}	C_{L-Arg} [mg]	U C_{L-Arg}
1.	0.45	1.5	1.04	0.2
2.	0.44	1.0	1.12	1.8
3.	0.40	1.0	0.98	1.0
4.	0.40	1.0	1.05	0.4
5.	0.44	1.0	1.04	0.2
6.	040	1.0	0.97	1.2
$\overline{X}$ ± SD	0.42 ± 0.02		1.03 ± 0.05	
SD	0.02		0.05	
RSD [%]	4.76		4.85	
S $\overline{X}$	0.01		0.02	
t	2.57		6.86	
P (%)	95.00		99.90	
$\overline{X}$ ± t . S $\overline{X}$	0.42 ± 0.03 (0.39 ÷ 0.45)		1.03 ± 0.14 (0.89 ÷ 1.17)	
E [%]	2.38		1.94	

2. Accuracy

The obtained results for the estimation of an analytical parameter accuracy by the degree of recovery R and RSD for the examined three concentrations of L – Glutamic acid in model mixtures (80 %, 100 %, 120 %) are correspondingly: 102.08 % ± 5.69 % (80 %); 99.67 % ± 1.90 % (100 %); 99.19 % ± □ 2.90 %.

3. Linearity

The proportional accordance between concentration (C) and peak height (H) is found and the coefficient of regression (R = 0.99746) is calculated.

4. Limit of detection (LOD) and limit of quantitation (LOQ)

By the applied HPLC method both for the analysed aminoacids L – Glutamic acid and L – Arginine are obtained the following data: limit of detection – C_{LOD} = 4.0 µg/ml; limit of quantitation – C_{LOQ} = 40.0 µg/ml.

Discussion

The identity of L – Glutamic acid in Tonotyl ® solution is confirmed by the relevance between sample and reference standard retention times as follows: in methanol:destilled water (1:1) – t_R = 5.333; in 20 mM solution of ammonium acetate – t_R = 2.4. The identity of L – Arginine in Tonotyl ® solution and in Sargenor ® amp. is shown by the same retention times like for reference standard of L – Arginine as follows: in methanol:destilled water (1:1) – t_R = 2.4; in 20 mM solution of ammonium acetate – t_R = 2.9.

From the results of analysis of Tonotyl ® solution is obvious that for both mobile phases the effectivity of resolution is estimated on the base of number of theoretical plates: N = 33390 in methanol:destilled water (1:1); N =13604 in 20 mM solution of ammonium acetate. From the presented results is obvious that the effectivity of resolution, obtained with using of methanol – destilled water (1:1) is better in comparison with the resolution, obtained with using of 20 mM solution of ammonium acetate.

From the results is shown that the selectivity factor (α) against L – Arginine is better in methanol – destilled water (1:1) (α = 2.22) than in 20 mM solution of ammonium acetate (α = 1.19). Inspite of better chromatographic parameters the application of mobile phase methanol:destilled (1:1) water is reduced, because of deviation of analytical parameters: low accuracy and low precision. This can be explained by the fact that during the time methanol cases noise of mobile phase.

Validation of HPLC method for analysis of L – Glutamic acid and L – Arginine.

For the development of the validation procedure of HPLC method are studied some analytical parameters such as: selectivity, precision, accuracy, linearity, limit of detection and limit of quantitation.

1. Selectivity (in respect of supplements) – the ability to assess unequivocally the analyte in the presence of components that may be expected to be present.

”Placebo” solutions, containing all supplements without the active substances are prepared at the same manner correspondingly like sample preparation of Tonotyl ® solution and Sargenor ® amp. The selectivity of the HPLC method is proved by the fact, that on chromatograms with ”placebo” preparations aren’t exist peaks with t_R responded to the respective t_R, obtained with standard preparations of L – Glutamic acid and L – Arginine.

2. Precision – expresses the closeness of agreement (degree of scatter) between a series of individual test measurements, obtained after applying the method repeatedly to multiple samplings of the same homogeneous sample under prescribed conditions. Repeatability (intra – assay precision) refers to apply the analytical procedure in the same laboratory, using the same analyst with the same equipment for a short period of time.

Six (6) equal homogenous samples from Tonotyl ® solution containing L – Glutamic acid and six (6) equal homogenous samples from Sargenor ® amp., containing L – Arginine are analyzed separately by the written HPLC method. For the estimation of an analytical parameter precision (repeatability) is used the uncertainty of the result, which is determined by standard

deviation (SD), relative standard deviation (RSD) and confidential interval ($\overline{X} \pm t.S\overline{X}$) (Dimov 1999).

From Table 1 is obvious, that all of the obtained experimental results suit respective confidence interval: 1) for quantity of L – Glutamic acid in Tonotyl ® solution: $\overline{X} \pm t.S\overline{X} = 0.42 \pm 0.03$ (0.39 ÷ 0.45) (SD = 0.02; RSD = 4.76 %); 2) for L – Arginine in Sargenor ® amp.: $\overline{X} \pm t.S\overline{X} = 1.03 \pm 0.14$ (0.89 ÷ 1.17) (SD = 0.05; RSD = 4.85 %).

For all of the obtained by HPLC results for the content of L – Glutamic acid and L – Arginine respectively in every sample is nessesary to estimate the Shovene's criterion (U), because when U for one value is higher than the rellevant standard Shovene's criterion (U St.), the data must be removed as anexpected. The statistical requirement for Shovene's criterion for six samples is: U St. = 1.73 (N = 6). From the presented data are obvious the relations: $U\ C_{L-Glu} < U\ St.$; $U\ C_{L-Arg} < U\ St.$, which confirm that all experimental data suit standard requirements and it isn't nessesary to remove anyone of them as anexpected.

3. Accuracy – expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

An analytical parameter accuracy is performed by HPLC method, checking of model mixtures, prepared from "placebo" of Tonotyl ® solution with adding of an active substance L – Glutamic acid in ratio from 80 % to 120 % of theoretical concentration. Every sample is analyzed three times. Accuracy is estimated on the base of degree of recovery R (presented as % obtained quantity by using HPLC method) ± RSD (%) – relative standard deviation. From the obtained results is shown that for L – Glutamic acid in model mixtures the degree of recovery is: 102.08 % (80 %); 99.67 % (100 %); 99.19 % (120 %).

4. Linearity – the ability of an analytical procedure to elicit test results that are directly or by a well – defined mathematical transformation proportional to the concentration (amount) of analyte in the sample.within a given range.

For the investigation of analytical parameter linearity are prepared six solutions of reference substance L – Glutamic acid, with increasing concentration and are analyzed by the written HPLC method. The results are putted into linearity regression analysis and the coefficient of regression (R = 0.99746) is calculated. There is no mistake of the linear proportion and they're no mistake of the dependence of the other constituents of the mixture.

5. Limit of detection (LOD) = $C_{LOD} = C_{min.}$ and limit of quantitation (LOQ).

The detection limit is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated as an exact value. The quantitation limit is the lowest concentration of analyte in a sample that can be determined with suitable precision and accuracy under the stated experimental conditions. Both for L – Glutamic acid and L – Arginine by HPLC method are achieved: $C_{LOD} = 4.0$ μg/ml; $C_{LOQ} = 40.0$ μg/ml.

Conclusion

An analytical procedure for the determination by HPLC methods of L – Glutamic acid and L – Arginine is developed. The applied analytical method is found to be selective, reproducible and accurate in appointed linear intervals. The most important parameters are accuracy and selectivity. The obtained results are important for validation of producing and storage procedures of food additives containing L – Glutamic acid and L – Arginine.

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