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Chemical stability of new neurotensin (8-13) analogues

S. Michailova[1], T. Dzimbova[2], K. Kalíkova[3], E. Tesařová[3], T. Pajpanova[2*]

[1]*Medical University of Varna "Prof. Dr. Paraskev Stoyanov", Faculty of Pharmacy, 55 Marin Drinov str., Varna 9002, Bulgaria*

[2]*Institute of Molecular Biology "Roumen Tsanev", Bulgarian Academy of Sciences, Acad. G. Bonchev str., block 21, Sofia 1113, Bulgaria*

[3]*Charles University in Prague, Faculty of Natural Science, Department of Physical and Macromolecular Chemistry, Albertov 2030, 128 43 Prague, Czech Republic*

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Hydrolytic stability of the peptides is one of the most important properties regarding their application in a practice. Early information on the stability is essential for the pharmacokinetic behavior in the body, for the storage conditions, the occurrence of toxic effects associated with its degradation products, etc. The purpose of this study was to evaluate the hydrolytic stability of synthesized NT(8-13) analogues under physiological conditions such as body temperature at 37ºC and physiological pH values of 1.2 (stomach), 7.4 (blood plasma) and 8.5 - 9.0(thin intestine).

Key words: neurotensin, canavanin, hydrolytic stability

INTRODUCTION

Neurotensin (NT) is a peptide consisting of 13 amino acids (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Try-Ile-Leu) that has been originally isolated from calf hypothalamus [1]. Like other neuropeptides, neurotensin has different functions. It is a neurotransmitter and neuromodulator in the central nervous system and a local paracrine hormone in the periphery, particularly in the gastrointestinal tract [2-5].

Three different receptors for neurotensin (NTS1, NTS2, and NTS3) have been cloned and studied thus far [6-8]. Among the known NT receptors, it has been shown that NTS1 is over-expressed in various relevant tumors, including ductal breast cancer and pancreatic tumors [9-12]. Structure-activity studies have demonstrated that the minimal sequence required for full biological activity is the C-terminal part NT(8–13) (Arg-Arg-Pro-Tyr-Ile-Leu) [13] and has therefore been selected as a lead structure for medicinal chemists [6, 14–18].

Application of the neurotensin, or any other endogenous peptide as clinical available drugs has been impeded by their relatively poor receptor selectivity, rapid degradation *in vivo*, and inefficient to penetrate the blood–brain barrier.

In addition, the pH value of the medium is one of the most important factors influencing the stability of the compounds and drugs, including those of the peptides also. The metabolism of the drugs often was associated with hydrolysis of an ester or amide bond, and its rate depends on the temperature and pH of the solution. The knowledge of "pH profile" of the compound helps us to define the values for which it is most susceptible to degradation. This also enables us to determine that pH value, which will provide optimum stability of the molecule and retains its structure and concentration over time.

We recently reported the synthesis of five new NT(8-13) mimetics, the pilot studies on their toxicity and central nervous activity [19]. The purpose of this study was to evaluate the hydrolytic stability of these neurotensin analogues in different values of pH, available physiologically.

EXPERIMENTAL

Peptide synthesis

Reagents, resins and Fmoc-amino acids used in peptide synthesis were purchased from Merck (Darmstadt, Germany) and Iris Biotech GMBH (Germany). Solvents of dimethylformamide (DMF) and dichloromethane (DCM) were purchased from Merck (Darmstadt, Germany). Electrophoretic experiments were performed using a BeckmanP/ACE (Beckman Coulter Inc., Pasadena, CA, USA).

Synthesis of all peptides was performed by the conventional and manual stepwise Fmoc solid-phase synthesis on 2-chlorotrityl chloride resin with substitution, 1.4 mmol/g. The coupling of each amino acid was performed in the presence of 3 mol excess of Fmoc-amino acid, 3 mol excess of N-hydroxybenzotriazole (HOBt), 3 mol excess of diisopropylcarbodiimide (DIC), and 5 mol excess of diisopropylamine (DIPEA) in dimethylformamide (DMF). Completion of

* To whom all correspondence should be sent:
E-mail: tamara@bio21.bas.bg

coupling reactions were monitored by the Kaiser test and the Fmoc groups were removed by adding 20% piperidine in DMF. The peptides were cleaved from the resin and the final deprotection was done in a cocktail containing trifluoroacetic acid (TFA), triisopropylsilane (TIPS), thioanisole, and water (92.5: 2.5: 2.5: 2.5). The crude peptides were precipitated into cold petroleum ether/diisopropyl ether (50:50). Then, the precipitate was dissolved in 10% CH_3COOH and desalted by gel filtration on a Sephadex G25. The chemical purity of peptides was characterized by RP-HPLC and capillary electrophoresis**.**

Analysis

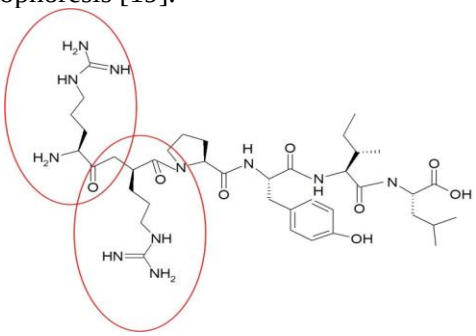
Purity: HPLC analysis were performed with LKB Bromma (Sweden) and **Waters** Alliance® (Waters Corporation, USA) instruments, variable detector using column: XTerra® MS C18, 3,5µm, 3.0 x 150 mm**;** eluent: ACN/0.05%TFA 5/95 (v/v), flow 0.4ml/min, 25°C, 220 nm, inj. volume 20 µL.

Stability: The hydrolytic stability of the peptides was determined by UV spectroscopy. For stability testing, a concentration $0.5.10^{-4}$ mol/l respectively of each of the peptides (NT - NT5) were dissolved in 10 ml of buffers: pH=1,2 (0.063 mol/l $HClO_4$); pH=7,4 (0.1 mol/l Na_2HPO_4 + 0.1 mol/l NaH_2PO_4); pH=9,0 (0.1 mol/l $Na_2B_4O_7$). The obtained solution of the peptides was tempered in the incubator ES-20 LKB (Sweden) at 37ºC. The testing samples were placed in a Beckman DU 650 spectrophotometer (Beckman Instruments, USA) equipped with a temperature-controlled cell changer; 1 ml quartz cuvettes were used. The decrease in the absorbance at 220 nm (UV maximum of the peptide) was monitored.

RESULTS AND DISCUSSION

The chemical stability of peptides is very dependent on amino acid composition and sequence. The two main cleavage bonds in the metabolic deactivation of NT(8–13) are Arg^8–Arg^9, Pro^{10}-$Tyr^{11}$and Tyr^{11}–Ile^{12}. To avoid such degradation, the terminal Arg units was replaced by canavanine (Cav), which was recently described as a non-proteinogenic Arg analogue. Because lysine was shown to be an attractive alternative basic residue [14], lysine replacement of the first/or second arginine moiety was also performed (Fig. 1.).

In this context our first objective was to synthesize the neurotensin mimetics in high yields and purities. Following purification by gel filtration on a Sephadex G25, peptides were isolated in high yields (86–91%), and excellent purities (>95%) confirmed by RP-HPLC (Fig. 2.) and capillary electrophoresis [19].



Code	Peptides
T0	Arg-Arg-Pro-Tyr-Ile-Leu
T1	**Lys**-**Cav**-Pro-Tyr-Ile-Leu
T2	**Cav**-**Lys**-Pro-Tyr-Ile-Leu
T3	**Cav**-**Cav**-Pro-Tyr-Ile-Leu
T4	Arg-**Cav**-Pro-Tyr-Ile-Leu
T5	**Cav**-Arg -Pro-Tyr-Ile-Leu

Fig. 1. Neurotensin (8-13) analogues.

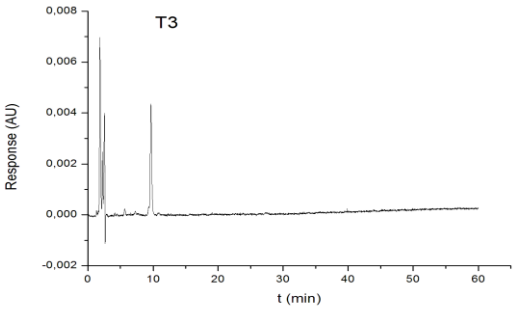


Fig. 2. RP-HPLC chromatogram at 220 nm of **Cav^8-Cav^9**-neurotensin (8-13) analogue

Therefore, in order to determine the hydrolytic stability of the synthesized neurotensin mimetics, we investigated their behaviour in three different physiological pH values of 1.2 (stomach), 7.4 (blood plasma) and 8.5 - 9.0 (thin intestine) using UV-spectroscopy and RP-HPLS as well. At defined intervals of the time we measured the absorbance at 220 nm. For each of the studied peptide has plotted graphics depending on time/concentration.

The hydrolytic stability of the native peptide Arg-Arg-Pro-Tyr-Ile-Leu (T0) is presented in Fig. 3. In acidic range the concentration of peptide remains constant during the first hour of the study and then gradually decreased. On the fourth hour it was under 40% and of the sixth hour zero. The stability of T0 in neutral and alkaline media was even smaller. On the sixtieth minute, the

concentration of T0 was under 50%, and of the third hour of experiment lower than 10%.

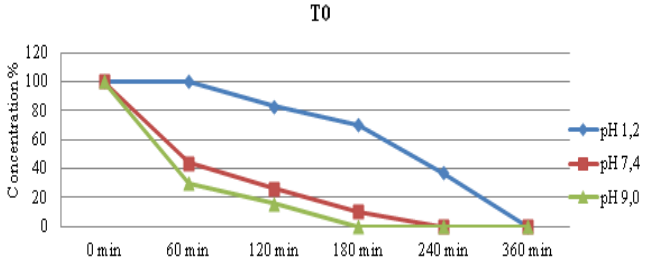


Fig. 3. Hydrolytic stability of T0 on different pH measured at 220 nm by UV – spectroscopy. As shown in Figure 4 at neutral pH, the peptide **Lys-Cav**-Pro-Tyr-Ile-Leu (T1) was stable for 60 minutes and the concentration was about 90%. Such a high stability at pH = 7,4 was observed only for the T3 analogue. Better stability in neutral zone, can significantly affect the biological potential of this NT analogue. The hydrolysis of peptide in acidic media goes slowly, as the concentration (80%) was maintained until the third hour. At the end of the experiment concentration of T1 was 40%. It is also evident from the graph, that the hydrolysis goes at a high speed at an alkaline pH (pH = 9.0).

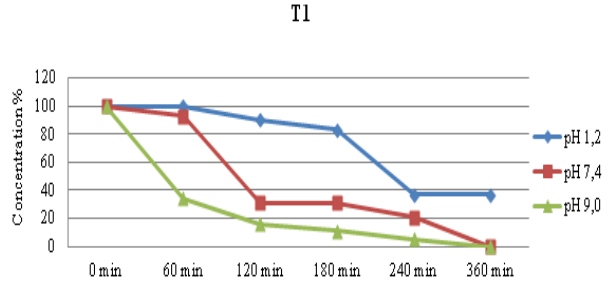


Fig. 4. Hydrolytic stability of T1 on different pH measured at 220 nm by UV – spectroscopy.

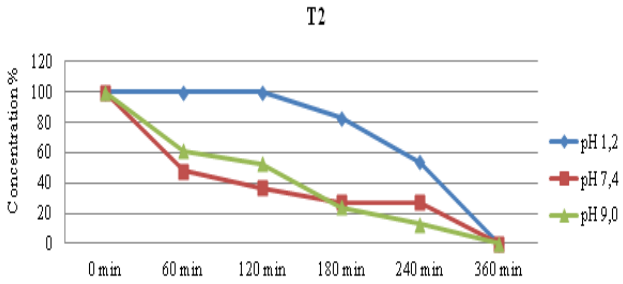


Fig. 5. Hydrolytic stability of T1 on different pH measured at 220 nm by UV –spectroscopy.

As shown in Figure 4 at neutral pH, the peptide **Lys-Cav**-Pro-Tyr-Ile-Leu (T1) was stable for 60 minutes and the concentration was about 90%. Such a high stability at pH = 7,4 was observed only for the T3 analogue. Better stability in neutral zone, can significantly affect the biological potential of this NT analogue. The hydrolysis of peptide in acidic media goes slowly, as the concentration (80%) was maintained until the third hour. At the end of the experiment concentration of T1 was 40%. It is also evident from the graph, that the hydrolysis goes at a high speed at an alkaline pH (pH = 9.0).

On the Figure 5 is visible that at pH = 1,2 the hydrolysis proceeds slowly up to 180 minute and the concentration of **Cav-Lys**-Pro-Tyr-Ile-Leu (T2) remained high (80%). After the third hour the concentration of the peptide decreased rapidly and the end of the way of expression drops to 0. At pH = 7.4 and pH = 9,0, absorption quickly decreases, indicating more rapid hydrolysis rate and a lower stability of the peptide. The dynamics of the two curves is similar.

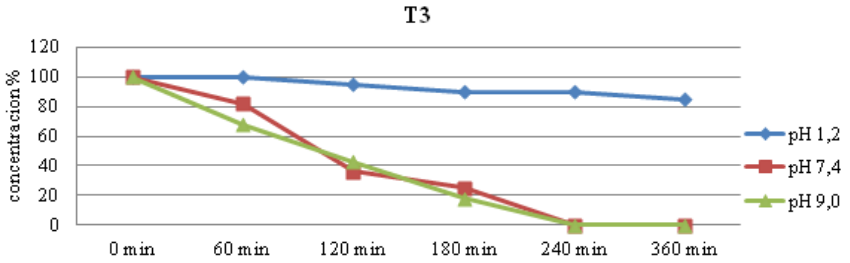


Fig. 6. Hydrolytic stability of T3 on different pH measured at 220 nm by UV –spectroscopy.

The stability of **Cav-Cav**-Pro-Tyr-Ile-Leu (T3) in the acidic pH (Fig. 6.) was the highest from the all tested NT analogues. The measured absorbance at 220 nm and pH 1.2 is slowly declining and the concentration remained relatively constant during the study, and on the sixth hour it was over 80%. A greater stability was observed for this peptide at pH = 7,4, in comparison to the other synthesized analogues. At neutral pH, it retained 90% of its concentration until the 60th minute, then hydrolytic stability gradually decreases. This stability in the neutral media may be very important at a later stage, when defining the route of administration of peptides and for testing its biological effects. The dynamics of the curve at pH = 9,0 also show better hydrolytic stability of T3 compared to the other analogues.

The compound Arg-Cav-Pro-Tyr-Ile-Leu (T4) was also unstable in alkaline and neutral condition. The process of hydrolysis goes at high speed for the first hour. On the 60th minute the concentration of the substance was 40% and 60% respectively. It

can be seen from the graph in Fig. 7. that in the solution with pH = 1,2 hydrolysis was more slowly than at pH = 7,4 and pH = 9,0, but the peptide T4 was less stable than T5 under the same conditions.

It can be seen on Fig. 8. that at the beginning of the study, the hydrolysis goes with a very high speed at the neutral (pH = 7,4) and alkaline (pH = 9,0) range. On the 60 minutes, the concentration of **Cav**-Arg -Pro-Tyr-Ile-Leu (T5) dropped almost in half in the alkaline solution and is only about 10% in the neutral. In the acidic range (pH = 1,2), the concentration of T5 remains relatively constant until the third hour of the study (90%), and then decreases gradually to 40% at the end of the measurement.

The chemical stability during storage represent possibility for any substance to left unchanged due to the influences of the type of an internal reaction or external factors such as air, heat, light and etc. All studied for hydrolytic stability peptides were stored at a temperature -10^{0}C for 1 year. Using UV-spectroscopy and analytical RP-HPLC we checked hydrolytic stability of the peptides at neutral and alkaline pH. It was found that they remained unaltered, as well as, their physical and chemical, and thus it can be considered to be chemically stable under these conditions.

For example we presented hydrolytic stability of the compound T3 at neutral and alkaline pH. In the previous experiment (Fig. 6.) the peptide T3 showed the highest stability in comparison with the other analogues. The rate of the hydrolysis and reducing concentration of determined peptide by HPLC are consistent with the data that we received by UV-spectroscopy. The better stability of T3 during the first hour in the neutral and alkaline pH was confirmed once again.

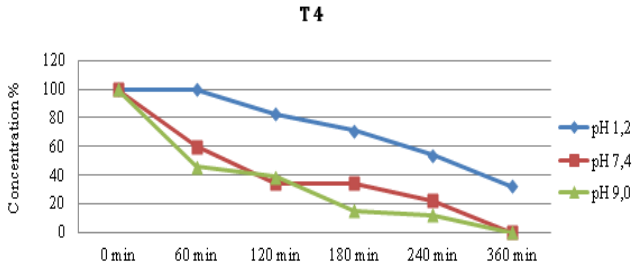


Fig. 7. Hydrolytic stability of T4 on different pH measured at 220 nm by UV –spectroscopy.

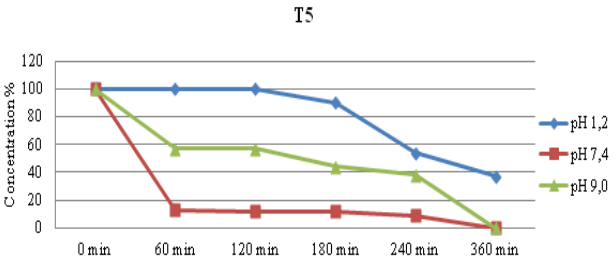


Fig. 8. Hydrolytic stability of T5 on different - pH measured at 220 nm by UV –spectroscopy.

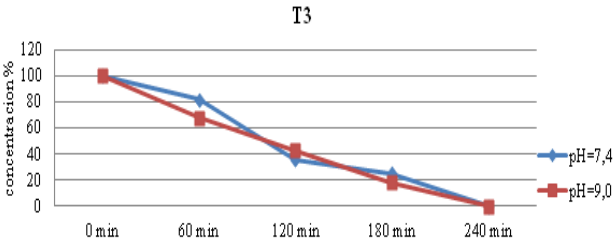


Fig. 9. Hydrolytic stability of T3 after 1 year storage measured at 220 nm by UV –spectroscopy.

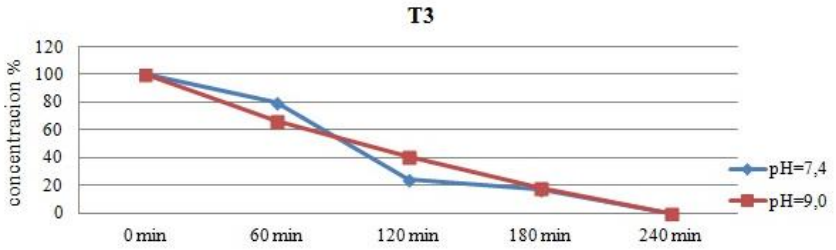


Fig. 10. Hydrolytic stability of T3 after 1 year storage measured at 220 nm by RR-HPLC.

The dynamics of curves presented in Fig. 9. is the same with that presented in Fig. 6. That gives us reason to conclude that T3 was stable even after 1 year of storage.

In addition the rate of the hydrolysis and reducing concentration of determined peptide by RP-HPLC are consistent with the data that we received by UV-spectroscopy. The better stability of T3 during the first hour in the neutral and alkaline pH was confirmed once again.

CONCLUSION

As a result of the experiments it can be concluded that:

• The dependence time / concentrations of T0 shows the low hydrolytic stability of native NT fragment and confirmed a need to develop a new, more stable analogues;

• The stability of the newly synthesized NT-analogues in acidic range is higher than that of the native (8-13) fragment of the NT;
•The hydrolysis of T3 in the acid buffer (pH = 1,2) takes place with very low speed, thereby retaining about 90% of its initial concentration. This gives us reason to believe that the analogue was stable at this pH;
• From all tested NT-analogues, compounds T1 and T3 showed the highest stability at neutral pH. On 60 minute their concentration is about 90%;
• The replacement of arginine residues with canavanine increased hydrolytic stability of the peptide analogues, and this is most likely due to the lower basicity of the oxy-guanidine group of canavanine compared to those of the guanidino group of arginine;
• The hydrolytic stability of the synthesized analogues was retained after one year of storage.

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ХИМИЧНА СТАБИЛНОСТ НА НОВИ АНАЛОЗИ НА НЕВРОТЕНЗИН(8-13)

С. Михайлова[1], Т. Дзимбова[2], К. Каликова[3], Е. Тежарова[3], Т. Пайпанова[2]

[1]Медицински университет „Проф. д-р Парашкев Стоянов", Варна, Фармацевтичен факултет, ул. „Марин Дринов" № 55, Варна 9002, България

[2]Институт по молекулярна биология „Акад. Румен Цанев", Българска академия на науките, ул. „Акад. Г. Бончев"бл. 21, София 1113,

[3]Карлов университет на Прага, Факултет природни науки, Катедра физико- и макромолекулна химия, „Албертов" 2030, Прага 128 43, Чехия

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(Резюме)

Хидролитичната стабилност на пептидите е едно от най-важните свойства по отношение на тяхното приложение в практиката. Ранната информация за стабилността е основна за фармакокинетичното поведение в тялото при условия на съхранение, появата на токсични ефекти, свързани с неговите разпадни продукти и т.н. Целта на това изследване е да се проучи хидролитичната стабилност на новосинтезирани пептиди при физиологични условия, като телесна температура при 37°C и pH стойности при 1.2 (стомах), 7.4 (кръвна плазма) и 8.5-9.0 (тънки черва).