HUPERZINE A: A PROSPECTIVE PROPHYLACTIC ANTIDOTE AGAINST ORGANOPHOSPHATE NERVE AGENTS. CAPILLARY ELECTROPHORESIS DETERMINATION

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Souhrn

Byla vypracována nová, vysoce citlivá CZE metoda stanovení huperzinu A, perspektivního profylaktického antidota proti vojensky významným nervovým plynům a perspektivního léčiva Alzheimerovy nemoci. Huperzin A je alkaloid izolovaný z čínské přesličky Huperzia serrata. Byla studována absorpční spektra látky a její elektroforetické chování. Látka vykazuje dvě absorpční maxima (λ_{max} 230 and 310 nm). Její disociační konstanta byla stanovena spektrofotometricky a pomocí kapilární zónové elektroforézy (CZE) a její hodnota je pK = 7,70 ± 0,07. Byla vyvinuta CZE metoda pro stanovení huperzinu A ve farmaceutických preparátech a krevním séru. Metoda je založena na separaci disociované formy huperzinu A, jejíž pohyblivost je 50,7 x 10^{-9} $m^2V^{-1}s^{-1}$. Byly stanoveny optimální separační podmínky: acetátový pufr (pH 4,60; 20 mM) jako elektrolyt, dělicí napětí 20 kV, teplota 25 °C, 10sekundová hydrodynamická aplikace látky na kolonu a detekce při vlnových délkách 230 a 310 nm. Analýzy byly prováděny v neupravované křemenné kapiláře délky 47,5 cm (39,0 cm k detektoru) a vnitřním průměru 75 μm. Detekční limit byl 70 mg huperzinu A. Metoda byla vyzkoušena u dvou farmaceutických preparátů a získané výsledky byly v dobré shodě s deklarovaným množstvím látky. Relativní přesnost metody je ± 2,30 %. Byla také vyvinuta metoda pro stanovení huperzinu A v krevním séru s detekčním limitem ≈ 110 μg/l, která je vhodná pro farmakokinetické a terapeutické monitorování této látky.

Klíčová slova: Huperzin A; Profylaktické antidotum proti organofosorovým nervovým látkám; Alzheimerova nemoc; Kapilární zónová elektroforéza.

Summary

A new, highly sensitive capillary zone electrophoresis method for determination of huperzine A, a perspective prophylactic antidote against militarily important nerve agents and anti-Alzheimer's disease drug, was developed. Huperzine A is an alkaloid isolated from the Chinese club moss Huperzia serrata. The absorption spectra and electrophoretic behaviour of the compound were studied. The compound shows two absorption maxima (λ_{max} 230 and 310 nm). The protonation constant was determined by spectrophotometry and capillary zone electrophoresis (CZE) and the recommended value is pK = 7.70 ± 0.07. A CZE method for the determination of huperzine A in pharmaceutical tablets and serum has been developed. This method is based on separation of the compound as a protonated species with electrophoretic mobility determined as 50.7 x 10^{-9} $m^2V^{-1}s^{-1}$. The optimal separation conditions were: acetate buffer (pH 4.60; 20 mM) as a background electrolyte, a separation voltage of 20 kV, a temperature of 25 °C, hydrodynamic injection 10 s and detection at wavelengths 230 and 310 nm. The analyses were run in an uncoated fused-silica capillary with a total length of 47.5 cm (39.0 cm to the detector) and the 75 μm I.D. Detection limit was found to be 70 μg/l. The method of huperzine A determination was applied for analysis of two pharmaceutical products. The results were found to be in good agreement with the declared amount. The precision of the method is ± 2.30 % rel. The method for determination of huperzine A in serum was also developed with a detection limit of ≈ 110 μg/l which is applicable for pharmacokinetics and therapeutic drug monitoring of this compound.

Key words: Huperzine A; Prophylactic antidote against organophosphate nerve agents; Alzheimer's disease; Capillary zone electrophoresis.

Introduction

Organophosphate nerve agents are very toxic synthetic compounds developed for use in chemical warfare. These substances are still considered as potential threats in both military or terrorism conflicts. Organophosphate agents are potent irreversible inhibitors of acetylcholinesterase (AChE), vital enzyme in central and peripheral nervous systems. Therefore, prophylaxis against nerve agents is based upon protection of AChE against irreversible inhibition by organophosphates. Partial inhibition of AChE by some reversible inhibitors protects the enzyme against irreversible inhibition by organophosphates and against the lethal effects of organophosphorous nerve agents. Well-tried reversible inhibitors are carbamates, for example physostigmine (Philippens et al., 2000; Tuominen and Hanninen, 1999) and namely pyridostigmine, very often with combination with some anticholinergics (Meshulam et al., 2001; Kassa and Vachek, 2002). Since pyridostigmine does not penetrate into the brain, it does not afford protection against seizures and subsequent neuropathology induced by an organophosphate agent such as soman. Physostigmine naturally penetrate into the brain and therefore physostigmine is more efective than pyridostigmine in protecting against the detrimental effects of soman (Miller et al., 1993). However, physostigmine is more toxic than pyridostigmine and the difference between its therapeutic and toxic dose is very narrow. New reversible inhibitors of acetylcholinesterase, suitable for prophylactic antidote development against organophosphate nerve agents are intensive seek.

One of these compound is huperzine A, unsaturated sesquiterpenic compound with a pyridone moiety and primary amino group, first isolated from the Chinese club moss *Huperzia serrata*. It is an interesting anticholinesterase compound from the traditional Chinese herbal medicine (Patocka, 1998). The moss has been used for treating fever, inflammation, schizophrenia and memory loss. As a modern herbal supplement, huperzine A is used therapeutically to treat Alzheimer's disease and other age-associated memory impairments. Huperzine A (Fig. 1) is a strong, centrally active, reversible inhibitor of cholinesterase with high selectivity to acetylcholinesterase (AChE) (Tang et al., 1989). Only (-) form of huperzine is biologically active. Huperzine A has been shown to penetrate the blood-brain barrier and it is more stable than the carbamates used as pretreatment for organophosphate poisoning. Huperzine A – AChE complex has a longer half-life than other prophylactic sequestering agents (Raves et al., 1997) and it has been proposed as a pretreatment drug for nerve agent toxicity by protecting AChE from irreversible organophosphate-induced phosphorylation (Grunwald et al., 1994; Patocka and Kassa, 1999). In this respect huperzine A is very perspective prophylactic compound against organophosphate agent poisoning (Lallement et al., 2002). For further progress in prophylactic antidote based on huperzine A, sensitive analytical methods are needed. From separation techniques only reverse phase HPLC have been used to determine huperzine A in plasma (Qian et al., 1995). Recently new sensitive and selective Capillary Electrophoretic (CE) method for the estimation of huperzine A has been developed and briefly reported somewhere (Pena-Mendéz et al., 2001). The aim of this work was therefore to study Capillary Zone Electrophoresis (CZE) behaviour of huperzine A as an important anticholinergic drug and if possible to develop reliable and rapid CZE method to determine huperzine A in pharmaceutical preparatives and biological fluids.

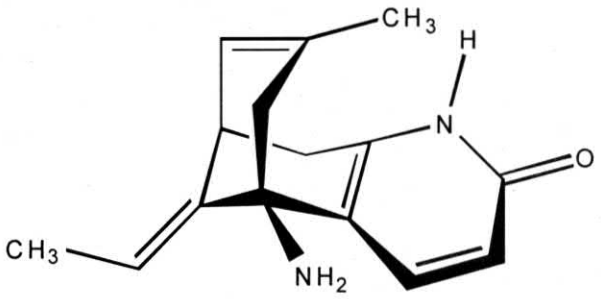

Fig. 1: Chemical structure of huperzine A, chemically 9-amino-13-ethylidene-11-methyl-4 azatricyclo [7.3.1.0(3.8)]trideca-3(8),6, 11-trien-5-one, empirical formula C15H18N2O, (M. W. 242.32), is an alkaloid isolated in 1982 from the Chinese plant Huperzia serrata by researchers at the Shanghai Institute of Materia Medica and Zhejiang Academy of Medical Science (Lin et al., 1986)

Experimental

Chemicals and Reagents

Sodium acetate and phosphate were of analytical grade from Lachema (Brno, Czech Republic). Hydrochloric acid and sodium hydroxide were from Merck (Darmstadt, Germany). All chemicals were of analytical grade and were used without purification. Double distilled water obtained from a quartz distillation stand of Heraeaus Quarzschmelze (Hanau, Germany) was used to prepare all the solutions. Mesityloxide used as an electroosmotic flow marker and benzimidazol were supplied from Fluka (Basel, Switzerland). Galanthamine was from Nivalin (Pharmachim, Bulgary). Standard pH buffers were from Institute of Serum and Vaccines (Prague, Czech Republic). Standard of (-)-huperzine A was

purchased from Panorama Research Inc. (Mountain View, CA, USA). Stock solution of huperzine A: a weighted amount of the standard compound was dissolved in hydrochloric acid (50 mM) and the solution was stored in refrigerator. The solutions were subjected to sonification using an ultrasonic cleaner of Branson (Shelton, CT, USA). The background electrolytes (BGE) were filtered through glass crucible S4 filters from Kavalier (Sázava, Czech Republic) and degassed before use.

Instrumentation

Spectrophotometer

ATI/UNICAM UV/Vis spectrophotometer UV2 from Unicam (Cambridge, Great Britain) in all spectrum measurements was used.

Capillary Electrophoresis

A ^{3D}Capillary Electrophoresis Instrument from Agilent Technologies (Waldbronn, Germany) equipped with diode array detection, operating on 3D-CE CHEM Station was used for huperzine A analyses. An uncoated fused-silica capillary of Watrex (Prague, Czech Republic) with total length of 47.5 cm (39.0 cm to the detector) and 75 μm I.D. was used. The background electrolyte (BGE) was acetate buffer (pH 4.60; 20 mM). Samples were injected hydrodynamically for 10 s. The separation voltage 20 kV was applied (the negative polarity at the detection side) and experiments were run at temperature 25 °C. Absorbance was monitored at 200, 230, and 310 nm and/or high speed scan of the spectra were used throughout the work. Mesityloxide (MSO) 0.1 % (v/v) was used to determine the electroosmotic flow (EOF) under the same experimental conditions as for analysis of the samples.

The capillary was washed daily for 5 min with double distilled water and 5 min with BGE. Between the runs the capillary was rinsed with BGE and double distilled water. The vials containing BGE were replenished after each injection.

An OP-208 precision digital pH-meter of Radelkis (Budapest, Hungary) and pH-sensitive combined glass electrode (Radelkis) were used for pH measurements.

For data evaluation STATGRAPHICS PLUS V.5 package (STSC, Inc., USA) and SQUAD program software were used (Leggett, 1985).

Sample preparation

Tablets were weighted, added 1 ml of methanol and 2.5 ml of double distilled water. This solution was sonicated for 10 min and then filtered through a filter with pore diameter 0.4 μm.

Serum was deproteinated by two different methods:

a) serum (200 μl) was mixed with acetonitrile (ratio 40:60, v/v) for 15 s; then, the mixture was centrifuged for 1 min at 4000 r.p.m. and the supernatant was taken for the analysis,

b) serum (200 μl) was mixed with dichloromethane (ratio 1:5, v/v) and after the centrifugation an aliquot of the organic phase was taken to a separate extraction tube and huperzine A was re-extracted from the dichloromethane phase into the aqueous one using 0.01 M HCl (Malovaná et al., 2001).

Results and Discussion

Spectrophotometry

The absorption spectra of a standard solution of huperzine A were measured in ethanol-water media (2.5 % ethanol, pH 6.86, I= 0.1 M KCl). Spectra show two absorption maxima at 230 and 310 nm, respectively. Fig. 2 shows the absorption spectra as a function of pH in the range 2.98–9.66. It follows from Fig. 2 that there is only a small shift from 306 nm to 310 nm for the second maximum. The values of pK were calculated using SQUAD program (Leggett, 1985), and pK = 7.50 ± 0.10 has been obtained. Standard deviation of absorbance equal to ± 0.0037 was obtained in the position of the best fit, which is in a good agreement with experimental un-certainty (≈ ± 0.004). However, the standard deviation of pK is higher than for electrophoretic data. The reason is that absorption spectra of neutral and protonated species of huperzine A are very similar (Fig. 2).

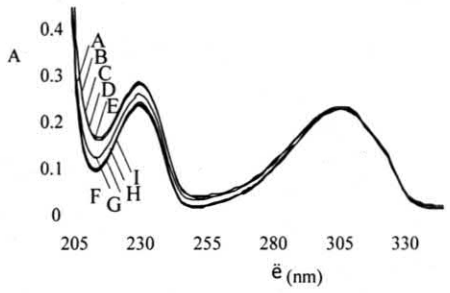

Fig. 2: The absorption spectra of huperzine A (7.3 μg/ml) as a function of pH: A (pH 2.98), B (pH 3.63), C (pH 4.34), D (pH 5.34), E (pH 5.87), F (pH 6.47), G (pH 6.86), H (pH 8.12), I (pH 9.66)

Capillary Electrophoresis

In order to optimize the electrophoretic conditions during the analysis different buffers at different concentrations, and pH values were tried. The dependence of effective electrophoretic mobility, μ_{eff}, on pH was studied and the results are shown for acetate buffer (20 mM). The structure of the compound presents one -NH_2 group, which can be proto-

nated in slightly acid media and thus cationic behaviour of huperzine A during the electrophoresis may be expected. At alkaline pH, the species is neutral.

If,

Huperzine A + H^+ $\leftrightarrow$ Huperzine AH^+ (A)

then μ_{eff}, the effective mobility of huperzine A is defined by the equation

$$\mu_{eff} = \mu_{HupA}\,[\text{Huperzine A}] + \mu_{HupAH^+}\,[\text{Huperzine AH}^+] \quad (1)$$

where μ_{HupAH^+} and μ_{HupA} are mobilities of protonated and neutral forms of huperzine A, respectively. Of course, mobility of neutral form is equal to zero. Thus, if

$[HupA] \ll [HupAH^+]$ then,

$$\mu_{eff} = \mu_{HupAH^+} \quad 2)$$

It can be seen from Fig. 3 that effective mobility, μ_{eff}, decreases when the pH is increasing. The pH value 4.60 was found suitable for the analysis. The effective mobility as a function of pH confirms cationic behaviour for huperzine A in acid solutions. At pH values greater than 5.5 the effective mobility of huperzine A starts to diminish and the compound is becoming to be neutral at pH > 8. The pK value of huperzine A protonation according to equation (A) was determined using CELET program (Havel and Janos, 1997) and value of pK = 7.70 ± 0.07 was obtained. Standard deviation is lower than that one obtained for spectrophotometry and because of the great similarity of huperzine A and huperzine A H^+ spectra, we do recommend this value (7.70) as more correct one.

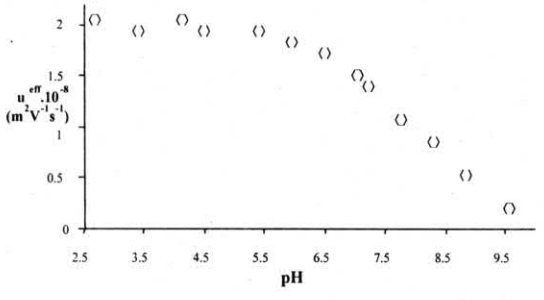

Fig. 3: Effective mobility of huperzine A as a function of pH, 15 µg/ml huperzine A

Evidently, for CZE determination of huperzine A the acid pH range (< 7) can be used. In order to optimize electrophoretic method, several BGE (phosphate, citrate, acetate) were tested. Different peak parameters (migration time, number of theoretical plates, symmetry, height, area, etc.) were evaluated, and finally acetate buffer (pH 4.60; 20 mM) was chosen as the optimal because it shows lowest noise, greatest peak height, and short migration time. The optimal conditions with good resolution, peak shapes and short time of analysis were: acetate buffer as a background electrolyte (pH 4.60; 20 mM), separation voltage 20 kV, temperature 25 °C, hydrodynamic injection for 10 s and detection at wavelengths 230 and 310 nm. The reproducibility of the migration time for 15 µg/ml huperzine A is shown in Fig. 4. A high reproducibility of the migration time was obtained for 10 repeated measurements of the HupA, the relative standard deviation value (RSD) was 1.30 %.

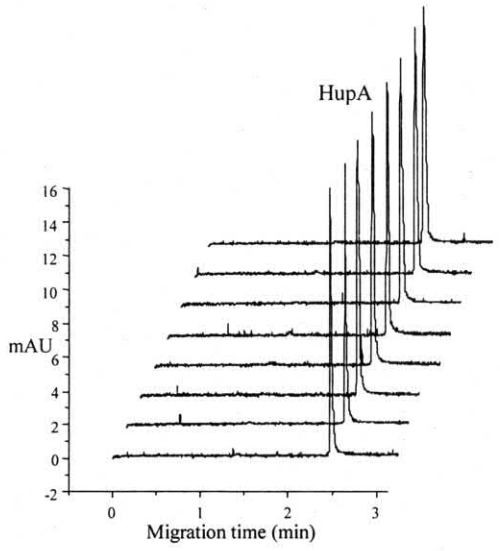

Fig. 4: Reproducibility study of the migration time of huperzine A, 15 µg/ml huperzine A

Using the optimal conditions described above, the use of the method for quantitative analyses was evaluated. For the calibration curve in the concentration range 2.6–10.3 µg/ml the following regression equation $y = 1.0357x + 0.01318$ was obtained, where x is the concentration of huperzine A. Correlation coefficient (R^2) is within the acceptable limit, $R^2 = 0.9976$. The limit of detection (LOD) for huperzine A in aqueous solution was estimated to be about 70 µg/l at a signal-to-noise ratio (S/N=3). The precision assessed for 15 µg/ml huperzine A standard solutions was ± 1.4 %.

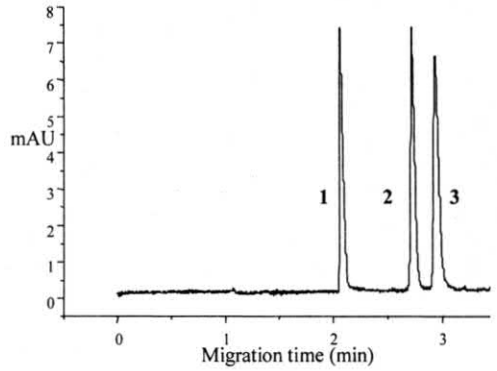

Fig. 5: The analysis of mixture (1) 16 µg/ml benzimidazole, (2) 16 µg/ml galanthamine and (3) 7 µg/ml huperzine A

Electrophoretic mobility of the protonated form of huperzine A was determined as 50.7 x 10^{-9} m^2 V^{-1} s^{-1}. Comparison with some other cationic drugs gives the following order: ammonium (72.2), pyridinium (51.1), huperzine AH^+ (50.7), tetramethylammonium (42.6), and tetraethylammonium (32.9 x 10^{-9} m^2 V^{-1} s^{-1}). An example of CZE analysis of the mixture of huperzine A with an other contra Alzheimer's disease drug such as galanthamine, etc. was done, while benzimidazol was used as a marker. Fig. 5 shows that migration time of protonated galanthamine is higher than migration time of huperzine A H^+. Protonated huperzine is migrating faster than galanthamine and its mobility is close to pyridinium.

Analysis of Tablets

The developed method was applied for the determination of huperzine A in commercial tablets. Two different types of tablets were analysed: a) The USA product – INTLECTS™ Huperzine A of Biomedisyn Corporation (Woodbridge, Connecticut, USA), b) Chinese tablet – Huperzine A Tablets® of Joyline & Joysun Pharmaceutical Stock Co. LTD. (China). The both products have the same declared content of 50.00 µg of HupA per tablet. The standard addition method was applied for the determination and the results of analysis are shown in Table 1. It was found that the huperzine A content determined either in USA or Chinese tablets was in a good agreement with declared content 50.00 µg per tablet.

Determination of Huperzine A in Serum

Huperzine A is used contra Alzheimer's disease and is recomendet to use against nerve gas poisoning. We have, therefore, examined also the possibility to determine huperzine A in serum. High concentration of proteins and ions in serum samples interfere

Table I

Assay on analysis of commercial pharmaceutical tablets

Tablet No.	INTLECTS ™ Huperzine A (USA)*	Huperzine A Tablets® (China)*
I	49.9 µg per tablet	49.2 µg per tablet
II	48.6	50.7
III	51.1	50.7
IV	48.4	48.8
V	50.5	48.7
Average	49.7	49.6
S.D.	1.2	1.0
RSD %	2.4	2.0

* declared amount 50 µg per tablet, S.D. standard deviation, RSD relative standard deviation

generally the analysis of small molecules. Two methods for deproteination were tested to eliminate interference from the matrix. Finally, the method developed by Malovana et al. (2001) was chosen. After the deproteination the eletrophoretic method described above was applied for the determination of huperzine A in serum. Example of an electropherogram concerning the determination of huperzine A in serum is shown in Fig. 6. The detection limit was estimated to be 110 µg/l (S/N=3).

When huperzine A was tested contra nerve gas poisoning, the research group of Ashani et al. (1994), Grundwald et al. (1994), Tonduli et al. (2001), and Lallement et al. (2002) estimated that the dose of 500 µg/kg was necessary to reduce toxicity due to soman. These authors studied effects of administration of huperzine A in different animals. In human adverse side effects at doses less than 500 µg/kg were not found (Xu et al., 1998). The applied method satisfies the required sensitivity for the determination of huperzine A values in serum for the administrated doses.

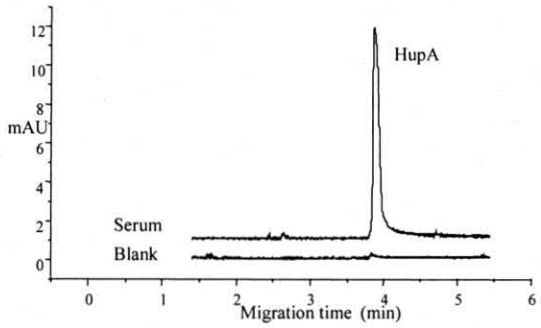

Fig. 6: An example of the electropherogram obtained during the analysis of 13 µg/ml huperzine A in the serum

Conclusions

Huperzine A is protonated in aqueous solution with pK ≈ 7.7 with very similar spectra of neutral and protonated forms. The protonated form of huperzine A, huperzine A H^+, has rather high electrophoretic mobility and this is the base of capillary electrophoretic method determination with a good detection limit (70 μg/l) at 230 nm. CZE method developed yields a good reproducibility and it was successfully applied for the determination in pharmaceutical products. It was also found that huperzine A can be determined in serum with sufficient detection limit (110 μg/l).

Acknowledgements

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