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Optimization of the capillary zone electrophoresis method for Huperzine A determination using experimental design and artificial neural networks

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Abstract

Huperzine A, natural product from *Huperzia serrata*, is quite an important compound used to treat the Alzheimer's disease as a food supplement and also proposed as a prospective and prophylactic antidote against organophosphate poisoning. In this work, simple and fast capillary electrophoresis (CE) procedure with UV detection (at 230 nm) for determination of Huperzine A was developed and optimized. Capillary electrophoresis determination of Huperzine A was optimized using a combination of the experimental design (ED) and the artificial neural networks (ANN). In the first stage of optimization, the experiments were done according to the appropriate ED. Data evaluated by ANN allowed finding the optimal values of several analytical parameters (peak area, peak height, and analysis time). Optimal conditions found were 50 mM acetate buffer, pH 4.6, separation voltage 10 kV, hydrodynamic injection time 10 s and temperature 25 °C. The developed method shows good repeatability as relative standard division (R.S.D. = 0.9%) and it has been applied for determination of Huperzine A in various pharmaceutical products and in biological liquids. The limit of detection (LOD) in aqueous media was 0.226 ng/ml and 0.233 ng/ml for determination in the serum.

Keywords: Huperzine A; Capillary electrophoresis; Experimental design; Artificial neural networks

1. Introduction

Huperzine A is an unsaturated sesquiterpenic compound with pyridone moiety and primary NH_2 group which was first isolated from the Chinese club moss *Huperzia serrata* [2] (cf. Fig. 1). The advantage of Huperzine A compared to the other compounds is its lower toxicity [1].

Huperzine A is known to be high selective to acetylcholine esterase (AchE). AchE destroys acetylcholine and terminates the nerve signal after it has been transmitted. In some of the memory disorders like Alzheimer's disease, acetylcholine is destroyed too fast. Only (−) form of Huperzine A is biologically active. Huperzine A has shown to penetrate the blood-barrier and it is more stable than the carbamates used as pretreatment for the organophosphate poisoning. Its complex with acetylcholine esterase has longer half-life than other prophylactic sequestering agents [2–4].

New approaches towards optimization of CE separation are using combination of experimental design (ED) and artificial neural networks (ANN). The use of multivariate methods (changing all parameters at the same time) is more correct and reliable but suitable ED must be applied in order to reduce the number of experiments. There are several types of experimental design, central composite design, factorial design, fractional factorial designs, and etc. [12]. The selection of a specific type of design depends primarily on both the nature and the extent of information we want to obtain. Further reduction of the experimental work can be reached by estimating the best separation conditions from ED experiments, either by response surface modeling or by soft modeling with the use of ANN, for example. This approach does not require a priori the knowledge of physicochemical constants or even understanding the separation mechanisms while an analytical method can be optimized effectively from a limited number of experiments.

Determination of Huperzine A by liquid chromatography methods has been published [6,11]. A simple assay based on

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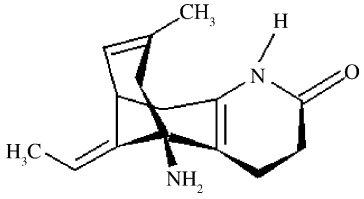

Fig. 1. Huperzine A structure.

the capillary electrophoresis (CE) has already been described for determination of Huperzine A [7]. The limit of detection (LOD) of this method for determination of Huperzine A had been estimated before, but it was not sufficient for biological fluids. Unfortunately, the method has not been optimized and validated. In this work, the factorial design with the central point combined with ANN was carried out in order to find the optimal electrophoretic conditions for determination of Huperzine A, using the acetate buffer. The method was applied to the quantitative analysis of pharmaceutical products. The method was optimized, validated and successfully applied to determination of Huperzine A in aqueous media and in serum.

2. Theoretical

2.1. Theory

Even though the use of artificial neural networks has been described in literature [5], short and brief theoretical information about ANN will be provided here. Artificial neural networks are widely used for optimization in separation techniques [5,8]. The theory of different networks has been reviewed, and defined ANN computing as the study of networks of adaptable nodes, which through the process of learning from task examples, store experimental knowledge and make it available for use. The node is the basic processing unit in neural network. ANN can consist of multiple layers of nodes arranged in such a way that each node in one layer is connected with each node in the next layer. The strength of the connection between two nodes is called weight, the weight is simply a real number. The set of weights values associated with neurons in the network determines the computational properties of the network, and training of a network is achieved by modifying these weights appropriately.

2.1.1. Architecture of the network

The architecture of the network is determined by the way the outputs of neurons are connected to the other neurons. In the standard architecture, the network neurons are divided into several groups called layers. All neurons (n) in a layer have the same number of inputs, the same number of weights, which will be modified during training. These inputs (m) come from neurons in the preceding layer of neurons or external input device. Thus, all neurons in the current layer simultaneously receive the m-variable input signal (X) from a set of m neurons of the preceding layer. All n outputs from the current layer are then simultaneously evaluated. In single monolayer architecture, these outputs form the final output of the networks; in a multilayer architecture, the n outputs are conveyed to the next layer, which is assumed here to contain p neurons.

Each layer has $n \times p$ weights and produces p outputs, which can again be propagated further to a deeper layer. The number of neurons in a layer and the number of layers depends strongly on the particular application: (1) on the number of variables for the objects, (2) on the number of objects involved in the study and (3) on the number and quality of answers sought in the study.

2.1.2. Training the network

What happens inside the network usually is (ignored), it is considered to be a black box with a number of inputs and outputs. After training, such a box should be able to achieve one of two tasks: (i) to yield for any given input signal the predefined output vector, (ii) or to activate for any input object. The aim is to find mapping between the group objects and the regions that are eventually formed in the plane of the output neurons of the networks.

2.1.3. Back propagation

Among different learning methods in the neural network computing, the most popular method is the Back Propagation method (BP) and it is often used in analytical applications. The back propagation learning algorithm does not reflect a particular similarity to real processes in brain, but has a sound theoretical background.

2.2. Experimental design combined with ANN

ANN has been used for modeling and optimization. Recently, ANN combined with the experimental design has shown great advantages that can be applied in the capillary zone electrophoresis. The approach of ANN–ED has several stages (i) performing a selected number of experiments using suitable experiments, (ii) learning stage, selection of ANN architecture that can approximate the data, and (iii) prediction of the optimal experimental conditions in order to reach the highest sensitivity of the determination [12].

The experimental design was a two-level factorial design with a central point. Thus, the experiments were carried out in all combinations of low/high buffer concentrations and low/high voltage and a central point (central composite design, CCD). The optimal ANN architecture was searched for, while the three-layer ANN with three nodes in the hidden layer was found optimal. As the input the buffer concentration and voltage were used and as the output either the peak area or the peak height or the migration time values were used (single output value). Validation was done in a limited number of additional experiments not included in the training set and good agreement between experiments and predicted

values proved the applicability of ANN structure. On the basis of the initial experiments, conditions of other experiments were predicted by ANN, experiments done and another, improved ANN model, was performed, etc. This hyphenated ED–ANN approach, as published for the first time [5] and used in several other communications [8] enabled to reduce the number of experiments and better analytical parameters (peak height) were achieved.

3. Experimental

3.1. Chemicals and products

(−) Huperzine A was purchased from Panorama research Inc. Mountain View (CA, USA). Mesityloxide used as the electroosmotic flow marker and benzimidazole were obtained from Fluka (Basel, Switzerland). Standard pH buffers were supplied from the Institute of Sera and Vaccines (Prague, Czech Republic). Methanol was purchased from Aldrich. The sodium acetate was of the analytical grade from Lachema (Brno, Czech Republic). Sodium hydroxide and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Double distilled water obtained from the quartz distillation stand of Heraeus Quarzschmelze (Hanau, Germany) was used for the standard and the sample preparation. Electrolyte solutions were filtered through a glass crucible S4 filters from Kavalier (Sázava, Czech Republic). Commercial tablets of Chinese HUPERZINE AND TABLETS of Joyline & Joysun Pharmaceutical Stock Co. Ltd. (Henan, China).

3.2. Equipment and capillary electrophoretic conditions

Ultrasonic bath from Branson (Shelton, USA) was used to sonicate the solution and to remove dissolved air. The OP-208 precision digital pH-meter of Radelkis (Budapest, Hungary) and pH-sensitive combined glass electrode (Radelkis) were used to measure pH. The CE experiments were carried out on SpectraPhoresis 2000 Thermo Separation Products (Fremont, CA, USA), which was driven by the CE software (version 3) operating under IBM OS/2 (version 1.2) and contained a programmable high-speed scanning multiple wavelength detector. Uncoated fused-silica capillaries were purchased from Watrex (Prague, Czech Republic) with the total length of 43 cm (35 cm to the detector) and the internal diameter of 75 μm. Burning off the polyamide coating on the capillary created a detection window. The detection wavelength was 230 nm with the rise time of 0.5 s. The detection was done at the cathodic side. Each day the capillary was rinsed for 10 min in 0.1 M NaOH, 10 min in water and for 2 min in the run buffer. Between each run the capillary was washed with 0.1 M NaOH for 2 min, followed by washing with water for 2 min, then flushing with buffer for 2 min at 25 °C and at the end of the day, the capillary was flushed with 0.1 M NaOH and water for 2 min.

3.3. ANN software and data processing

ANN software was purchased from the Trajan program Neural Network Simulator releases 3.0 D (1998; Trajan Software, Durham, UK) and processed on a Pentium personal computer.

4. Results and discussion

4.1. Development of CE method

Several background electrolytes (BGE) such as phosphate, formate, acetate, etc. were tested for (CE) analysis. Different peak parameters (migration time, height, and area, etc.) were followed. The highest peaks were obtained at acidic pH (4.6) using 20 mM acetate buffer. Cationic behavior at acidic pH value was expected for Huperzine A, due to protonation of amine group in the molecular [7]. Setting up the optimal CE method for determination of analyte requires consideration of a number of parameters to be optimized, such as voltage, pH, and the buffer concentration in order to have good high sensitivity measured as the peak area/migration time ratio (*A*/mt) and/or peak height. Experimental design can be used to obtain good description and prevision of the considered problem in order to find the optimum. The experiments were planned in order to homogeneously cover the experimental space [5].

In this work, the background electrolyte concentration and the separation voltage were used as input parameters, the output parameters were the peak height, peak area, and migration time of Huperzine A. In the preliminary experiments, buffer acetate was selected as the background electrolyte. Concerning the investigated experimental space, the voltage ranged from 10 kV to 25 kV, the acetate buffer concentration ranged from 5 mM to 60 mM. With the ANN optimum conditions, 10 kV separation voltage, using 50 mM acetate buffer as BGE, pH 4.6, at 25 °C, and injection time 10 s were chosen

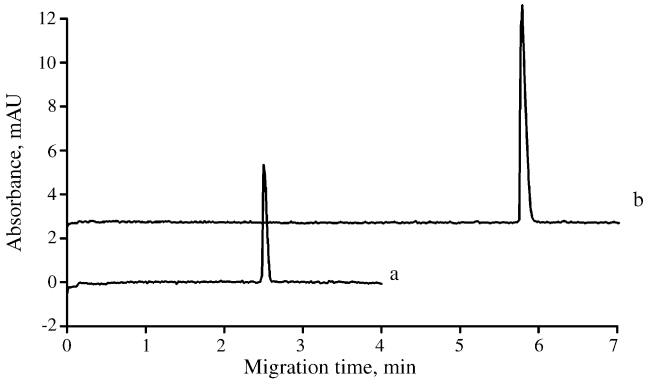

Fig. 2. Example of Huperzine A electropherograms (14.5 μg/ml) under conditions: (a) 20 mM acetate buffer, pH 4.6, voltage 20 kV, hydrodynamic injection 10 s, temperature 25 °C. (b) ANN optimized method: 50 mM acetate buffer, pH 4.6, voltage 10 kV, hydrodynamic injection 10 s, temperature 25 °C.

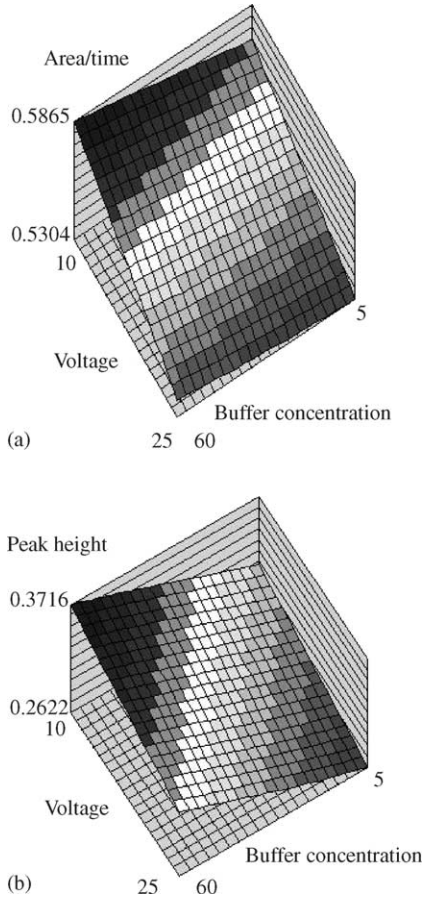

Fig. 3. Huperzine A peak area/migration time ratio (a) and (b) peak height as a function of buffer concentration and voltage.

as optimal conditions (Fig. 2). In particular, the response surfaces were drawn keeping two factors at a time at their central value, the third dimension being represented by the response (Fig. 3). Fig. 3a and b show that to maximize the response *A*/mt and the peak height, it is necessary to move towards high level of buffer concentration and low level of voltage. Validation of the modules around the optimized condition was carried out, verifying that there was close agreement between the predicted and measured response. After applying the optimal separation parameters as predicted by the artificial neural networks approach, the improvement of the sensitivity of the method was observed (Fig. 4). The reached detection limit

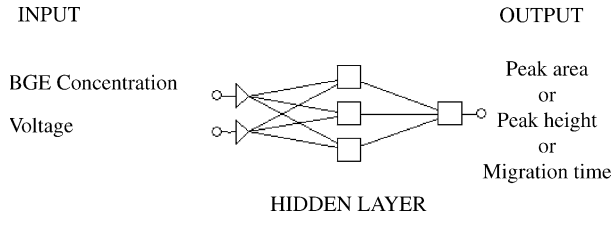

Fig. 4. An example of optimal ANN architecture used to optimize the Huperzine method. Input: concentration of BGE, voltage. Output: peak height, peak area, and migration time.

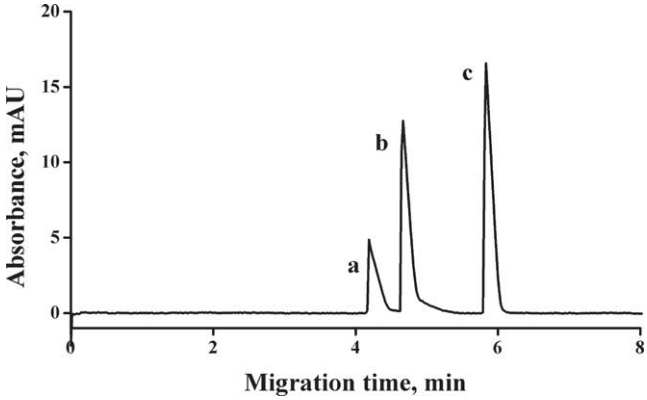

Fig. 5. The analysis of the mixture of (a) benzimidazole (9 μg/ml); (b) pyridinium (15 μg/ml); (c) Huperzine A (25 μg/ml) under conditions: 50 mM acetate buffer, pH 4.6, voltage 10 kV, hydrodynamic injection 10 s, temperature 25 °C.

was 0.226 μg/l in the aqueous media and 0.233 μg/l in the serum.

4.2. Validation

Validation was performed with respect to specificity, linearity, range, accuracy, the limit of detection (LOD), limit of quantitation (LOQ), and repeatability.

4.2.1. Specificity

The method indicated a significant degree of specificity as good selectivity in separation. Stability tests under long-term and accelerated storage conditions as requested by ICH [9] were done. Huperzine A, benzimidazol, and pyridinium were well separated (Fig. 5). Benzimidazol and pyridinium were used as markers and also for the comparison purposes. We also tried to use them as internal standards. However, the use of these two compounds (for example the peaks ratio) has not substantially improved the precision of analysis.

4.2.2. Linearity and calibration curves

Calibration curves were obtained by plotting the analyte concentration ratio versus the corrected peak area. The curve for Huperzine A in aqueous solution was evaluated across the range 20–250% of the test concentration 8.8×10^{-6} M, whereas the curve for Huperzine A in the serum was achieved across the range 30–200% of the test concentration 7.9×10^{-6} M. The linearity for Huperzine A was assessed in a wider range (from 1.76×10^{-6} M to 7.0×10^{-7} M) for Huperzine A, while six different concentrations of Huperzine A were prepared by diluting the standard stock solution. Applying the optimized conditions, the linear relationship for determination Huperzine A was found. The equation found was $y = 0.1371x + 0.0168$ ($n = 6$) with square of correlation coefficient equal to 0.9998. The calibration curve with five points was also tested for the spiked serum. The tested concentration range was from 2.7×10^{-6} M to 15.8×10^{-6} M.

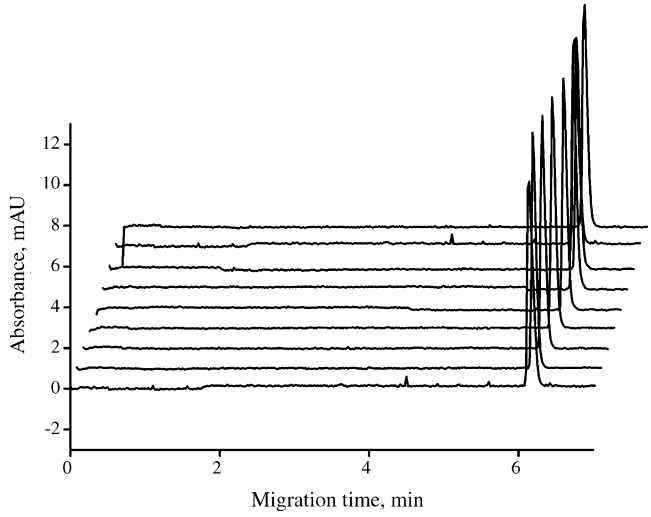

Fig. 6. Repeatability of Huperzine A determination (14.5 μg/ml) under conditions: 50 mM acetate buffer, pH 4.6, voltage 10 kV, hydrodynamic injection 10 s, temperature 25 °C.

The calculations were performed using the Excel software and the linear regression parameters obtained for the spiked serum were $y = 0.1219x - 0.0246$ ($n = 5$). The linearity was found to be good and acceptable for determination of Huperzine A.

4.2.3. Accuracy

Accuracy of the method was demonstrated as percent recovery in three samples at wide intervals of concentration levels and with three replicates each. The recovery was calculated using the standard solution at each level. The recovery of Huperzine A was found to be between 99% and 101% (R.S.D. = 1.1%) at 8.7×10^{-6} M level sample, 99–100% (R.S.D. = 0.3%) at 3.5×10^{-7} M level sample, and 100–102% (R.S.D. = 1.1%) at 6.0×10^{-7} M level sample. The results obtained for accuracy were found to be in good agreement for the method as well as the percent recovery.

4.2.4. Precision

The method precision was determined by measuring repeatability of relative migration times and normalized peak areas for Huperzine A in order to determine the repeatability of the method, replicate injections ($n = 9$), Huperzine A samples. The relative standard deviations (R.S.D.) were calculated for the migration time and the peak area ratio. Repeatability was better than 1% for the migration time and the peak area ratio (Fig. 6).

4.2.5. LOD and LOQ

For determination of LOD, solutions with low concentrations were injected in order to find the concentration with corresponding signal-to-noise ratio 3:1. The low detection limits were reached 0.226 ng/ml in the aqueous media and 0.233 ng/ml in biological fluid. The limit of quantitation was estimated by considering the amount of analyte, which gave the signal-to-noise ratio 3:10. The limit of quantitation was 0.69 ng/ml in aqueous media and 0.71 ng/ml serum (carried out according to ICH guidelines) [9,10].

Table 1
Assay on analysis of commercial pharmaceutical tablets of Joyline & Joysun pharmaceutical stock Co. Ltd. (Henan, China)

Tablets No.	Huperzine A	
	Found (μg)	Nominal (μg)
1	47.2	50
2	47.8	50
3	47.8	50
4	49.9	50
5	49.5	50
Average	48.4 ± 1.2[a]	50

[a] Standard deviation (S.D.).

4.3. Application

4.3.1. Determination of Huperzine A in pharmaceutical products

The tablets were separately weighed, and each one dissolved in 1 ml methanol, and 2.5 ml double distilled water. The solutions were sonicated for 10 min and transferred into the 10 ml volumetric flask, then filtered through a filter with pore diameter 0.45 μm. The method was applied for the analysis of commercial tablets of Huperzine A Chinese Co. The product has declared content of 50 μg per tablet. The results of analysis are shown in Table 1. The Huperzine A determined in this product was in good agreement with the declared content.

4.3.2. Determination of Huperzine A in the serum

Several methodologies for blood or serum samples preparation are reported in the literature [7,13,14]. It is usually necessary to deproteinize the serum samples. We have examined several possibilities. As the best possibility, using an organic solvent such as chloroform, dichloromethane, acetonitrile or ethyl acetate as the denaturation agents were found to be the best. In agreement with the literature [7] we proved that dichloromethane is optimal. After re-extraction, the centrifugation of the solution was applied. The supernatant was then used for the re-extraction. The procedure: serum was spiked with different concentrations of Huperzine A and deproteinated by mixing 1 ml of serum with dichloromethane (ratio 1:5, v/v) and shaking for 5 min, then centrifugated for 5 min. An aliquot of the organic phase was taken to a separation funnel and Huperzine A was re-extracted from the dichloromethane phase into the aqueous phase using 0.01 M HCl. A similar procedure was successfully used for determination of esmolol [8]. To conclude, determination of Huperzine A in the serum was done by the deproteination method as described above. After the extraction, when applying the optimized method, a good limit of detection (0.233 ng/ml) was reached.

5. Conclusions

The use of the experimental design combined with the artificial neural networks proved to be effective for optimization of CE Pharmaceutical product determination of Huperzine A in aqueous media and in a biological fluid. The method was optimized and validated with regard to specificity linearity, range, limit of detection quantitation and precision. Lower limit of detection was reached than the reported one [7]. The method was satisfactorily applied to assay the Chinese commercial product.

Acknowledgements

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