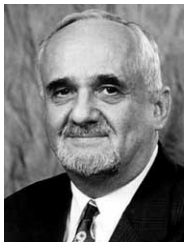
Analysis of Pralidoxime in Serum, Brain and CSF of Rats

Huba Kalász[1,2,#,*], Éva Szökő[3], Tamás Tábi[3], Georg A. Petroianu[1], Dietrich E. Lorke[4], Abdulrab Omar[5], Salem Alafifi[5], Almerri Jasem[5] and Kornélia Tekes[3]

[1]*Department of Pharmacology & Therapeutics, Faculty of Medicine & Health Sciences, United Arab Emirates University, Al Ain, P.O.Box 17666, United Arab Emirates;* [2]*Department of Pharmacology & Pharmacotherapy, Semmelweis University, Budapest, P.O.Box 370, Hungary 1445;* [3]*Department of Pharmacodynamics, Semmelweis University, Budapest, P.O.Box 370, Hungary 1445;* [4]*Department of Anatomy, Faculty of Medicine & Health Sciences, United Arab Emirates University, Al Ain, P.O.Box 17666, United Arab Emirates;* [5]*Faculty of Medicine & Health Sciences, United Arab Emirates University, Al Ain, P.O.Box 17666, United Arab Emirates*



Abstract: After administration of various amounts of pralidoxime to rats, the levels in serum, brain and cerebrospinal fluid (CSF) were measured using capillary zone electrophoresis (CZE). The calibration curves were established using spiked samples. The calibration covers the ranges from 0.3 – 200 μg/mL, 0.3 – 7 μg/mL and 0.1 – 7 μg/mL for serum, brain and CSF, respectively.

The CZE measurement opens the way to the fast and reliable determination of pyridinium aldoxime concentrations in serum, cerebrospinal fluid and brain, thereby monitoring blood-brain and blood-CSF penetration of pyridinium aldoxime-type antidotes clinically used in organophosphate poisoning.

Key Words: CZE, pralidoxime, serum, cerebrospinal fluid (CSF), brain.

[#]**Author Profile:** Huba Kalász was graduated as chemist, earned PhD, D.Sc. (chemistry) and med. habil (pharmacology). His research activity deals with the fate of drugs in the body: pharmacokinetics and metabolism using HPLC, HPLC-MS, capillary electrophoresis and computer assisted (in silico) methods. He works at Department of Pharmacology, Semmelweis University, Budapest, Hungary.

INTRODUCTION

The World Health Organization reports a high incidence (over 3 million cases per year) of accidental or intentional organophosphate poisoning [1]. The mechanism of toxicity is inhibition of esterases (mainly acetylcholinesterase), by covalent binding of the organophosphate to the serine residue at the active site of the enzyme [2]. The Tokyo Metro attack in 2000 [3] is evidence for the misuse potential of organophosphates and phosphonates.

This highlights the need for new and more effective antidotes. Pyridinium aldoximes are able to reactivate acetylcholinesterase by dephosphorylating the enzyme. The first oxime-type esterase reactivator to be introduced in the therapy of organophosphate poisoning was pralidoxime [4] (Fig. **1**); the substance is still used mainly in the United States [5]. Other widely used pyridinium aldoximes are trimedoxime (TMB-4), obidoxime, HLö-7 and HI-6: they are administered in combination with a muscarinic antagonist (most commonly atropine) [5]. Kuca *et al*. have synthesized several bis-pyridinium compounds with promising pharmacological activity [2, 6].

*Address correspondence to this author at the Department of Pharmacology & Therapeutics, Faculty of Medicine & Health Sciences, United Arab Emirates University, Al Ain, P.O.Box 17666, United Arab Emirates; E-mail: drkalasz@gmail.com

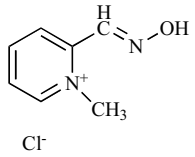


Fig. (1). The chemical structure of pralidoxime (PAM chloride).

Analysis of pyridinium aldoximes has been performed using mainly HPLC [7-12] monitoring with either UV [7-10] or electrochemical detector [11, 12]. A recent publication describes the determination of urinary pralidoxime using high-voltage capillary zone electrophoresis (CZE) [13]. CZE offers the advantage of both high sensitivity and selectivity, to a degree comparable to the most advanced recently described HPLC technique [14]. The analytical methods available for pyridinium aldoximes have been reviewed by Csermely *et al*. [15].

The blood-brain penetration of pyridinium aldoximes is an important issue, from both practical and theoretical points of view, since one of the postulated effects of pyridinium aldoximes is reactivation of the enzyme in the brain (for review, see [16]). Moreover, pyridinium aldoximes are highly hydrophilic compounds [16-18] so that, based on their lipophilicity, penetration through the blood-brain-barrier (BBB) is not likely [16].

1573-4064/09 $55.00+.00

Sakurada *et al*. [19] determined pyridinium aldoxime levels in the blood and in the brain using the HPLC-UV method. More recently, HPLC-MS-MS [14] was used to investigate the blood-brain barrier penetration of several pralidoxime analogues. These papers outline an experimental setting requiring the collection of brain dialysate for a relatively long period (1 hour). This appears to be problematic since the peak (maximum) level of pralidoxime is already reached after several minutes (less than 5 minutes following i.m. administration), and the oxime concentrations in serum and brain drop to very low levels over 60 minutes.

Our aim was to expand determination to the parallel measurements of pralidoxime level in serum, brain and CSF at the same time, and to analyze the penetration dependence on the pralidoxime dose.

MATERIALS AND METHODS

Materials

Pralidoxime (pyridine-2-aldoxime methochloride, 2-PAM chloride), β-alanin and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal Treatment

Male Wistar rats from United Arab Emirates University closed breeding colony weighing 179.0±17.8 g (mean±SD), were intramuscularly (i.m.) injected with various doses of pralidoxime. Rats were randomly assigned to one of the six groups of five rats each. Rats in groups 1-5 were administered 100, 50, 20, 10, and 1 μmol pralidoxime, respectively. 0.2 mL of the pralidoxime solution was injected intramuscularly to the hind limb. Controls (group 6) received only the vehicle. After 5 min, the rats were anesthetized using diethyl ether; serum and CSF were taken according to the standard procedures [20].

Briefly, animals under ether anesthesia were exsanguinated through the *medial canthus.* The samples were kept at 4 °C for 1 hour for clotting and the serum was subsequently separated by centrifugation (1600 g, 15 min, 4 °C). Cerebrospinal fluid (CSF; 90-140 μL) was drawn through the *foramen occipitale magnum* and collected in Eppendorf vial. The rats were sacrificed by decapitation, brain was dissected immediately on an ice-cold aluminum surface and weighed. The samples were kept frozen at -80°C until further treatments.

Sample Preparation

Serum and CSF samples were diluted fivefold with distilled water.

Brain samples were homogenized in four times the volume of their wet weight in distilled water. The insoluble tissue debris was removed by centrifugation at 20,000 g, 4 °C for 5 min. The supernatant was mixed with ice cold chloroform to extract lipids and to precipitate protein. After centrifugation at 20,000 g, 4 °C for 5 min the aqueous phase was used for the electrophoretic analysis.

Instrumentation and Determinations

All separations were performed with a P/ACE MDQ capillary electrophoresis system equipped with an UV detector set at 280 nm (Beckman-Coulter, Fullerton, CA, USA). Separations were carried out in fused silica capillaries of 75 μm ID, 365 μm OD, 40 cm total length, 30 cm to the detector (Polymicro Technology, Phoenix, AZ, USA). The background electrolyte used was 50 mM β-alanine – acetic acid, pH 4.0. The capillary was washed successively with 0.1 M sodium hydroxide, water and the separation buffer between runs. Samples were introduced into the capillary by pressure. The separations were carried out applying constant current of 22 μA (corresponding to 20 kV in the separation buffer), and the cartridge temperature was maintained at 15 °C.

The determinations were validated as detailed in our previous publications [21-23].

RESULTS

The samples were concentrated using transient isotachophoresis (ITP) at a moderately acidic (pH 4.0) background electrolyte to suppress the electroosmotic flow. Using this electrolyte system allowed about five times larger injection volumes compared with conventional capillary electrophoresis. The serum samples were diluted five-fold with distilled water to decrease protein and salt content.

Fig. (**2**) shows the electropherograms using conventional and large volume injection of spiked serum samples. The sensitivity is increased considerably without any deterioration in the separation efficiency in case of large volume injection.

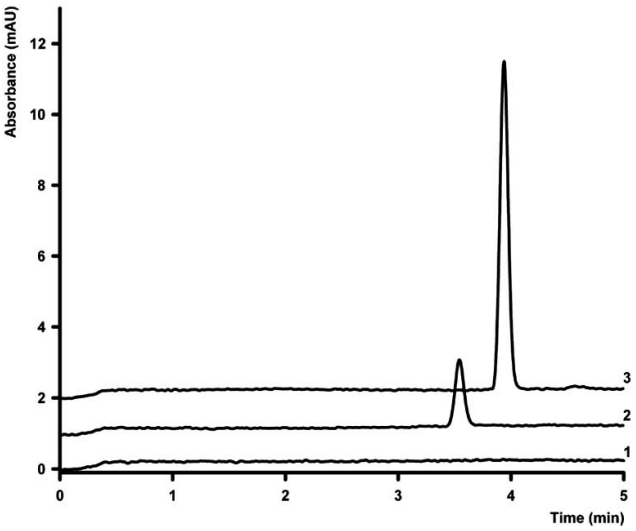


Fig. (2). Electropherograms of blank and spiked serum samples: blank serum sample (lane 1) and blank serum sample spiked with 10 μg/mL pralidoxime diluted fivefold with distilled water and analyzed by conventional (lane 2) and large volume injection (lane 3). Separation conditions are given in the experimental section. Sample was introduced into the capillary by pressure, applying 0.3 psi for 5 sec and 1 psi for 10 sec for conventional and large volume injection, respectively.

Fig. (**3**) shows the electropherogram of blank and spiked CSF samples. Similar methodical approaches were used; however, the different sample matrix allowed an even higher injection volume.

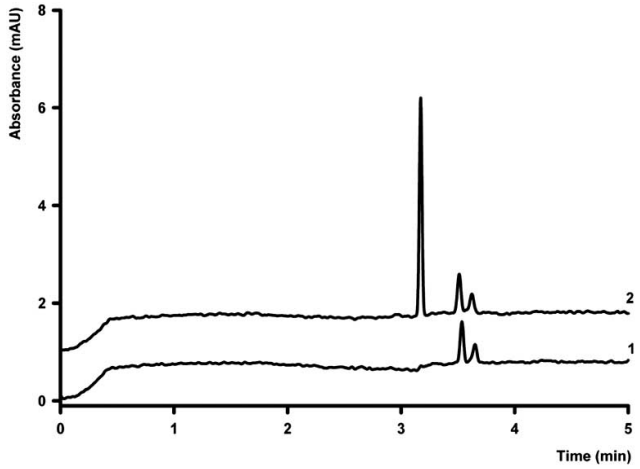


Fig. (3). Electropherograms of blank and spiked samples of the cerebrospinal fluid (CSF): blank CSF sample (lane 1) and blank CSF sample spiked with 3 μg/mL pralidoxime (lane 2) diluted five-fold with distilled water. Separation conditions are given in the experimental section. Sample was introduced into the capillary by pressure, applying 1.5 psi for 10 sec.

For brain extracts, a clean-up from the high lipid and protein content was necessary. Following homogenization in distilled water, the proteins and lipids were removed using centrifugation and extraction with ice cold chloroform, respectively. The electropherograms obtained by injection of blank and spiked brain samples are shown in Fig. (**4**).

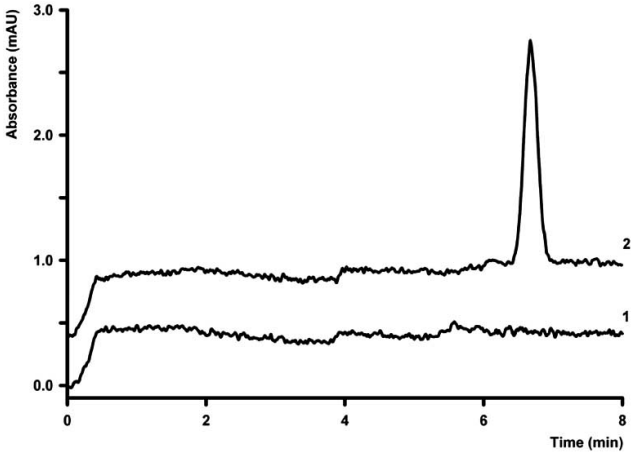


Fig. (4). Electropherograms of blank and spiked brain samples: blank brain sample (lane 1) and blank CSF sample spiked with 1 μg/mL pralidoxime (lane 2) homogenized in four volume of distilled water, mixed with ice cold chloroform and centrifuged at 20000 g, 4 °C for 5 min. Separation conditions are given in the experimental section. Sample was introduced into the capillary by pressure, applying 1 psi for 10 sec.

Calibration lines for each sample matrix were constructed by using spiked blank samples. Linearity was found through the range of 0.3-10 μg/mL, 0.1-7 μg/mL and 0.3-7 μg/mL of pralidoxime content, for serum, CSF and brain samples. The limit of detection values with signal to noise ratio of 3 were 0.1 μg/mL, 0.05 μg/mL, and 0.1 μg/mL for the three sample matrix, respectively.

Results of determinations are given in Table **1**. The standard deviation (SD) values were acceptable.

The ratios of the brain/serum and CSF/serum concentrations mirror dose dependence of pralidoxime penetration across the blood-brain- and blood-CSF barriers. When smaller doses were administered, the drug levels reached the central nervous system as a proportion of the amount injected were higher (Fig. **4**).

DISCUSSION

The quaternary amine pralidoxime bears permanent positive charges; thus it can be separated towards the cathode in a wide pH range. Sample induced transient ITP is an attractive way of improving detection sensitivity in case of biological samples, where otherwise the high salt content impairs the separation efficiency and limits the injection volume [22-25]. During isotachophoresis the uneven distribution of the electric field forces the sample ions to arrange their velocity to that of the fastest moving ion, the leading ion. The slower ions stack behind and migrate with the velocity of the leading ion. After dispersion of the leading zone the sample zones also destack and are separated by capillary zone electrophoresis [22]. During sample induced ITP, the high sodium content of the biological sample can be used as leading ion, while the co-ion of the background electrolyte should be chosen to serve as terminating ion [22, 23].

In this study we used β-alanine buffer. This zwitterionic compound possesses a partial positive charge at pH 4.0 resulting in low electrophoretic mobility fitting with the requirements of being a terminating ion. The sodium content of the serum samples served as leading ion. No sample preparation other than dilution is required.

Pyridinium aldoximes have the ability to reactivate cholinesterases inhibited by exposure to organophosphates [6, 16]. One of the selection criteria of aldoximes is their ability to pass the blood-brain barrier, and to achieve significant and therapeutically relevant concentrations in the central nervous system [16]. However, data on the brain and CSF concentrations of pralidoxime are scarce. Analytical monitoring of the brain penetration of pralidoxime was done by Sakurada *et al*. [14,19]. HPLC-UV [19], and HPLC/MS/MS [14] were used.

Houzé *et al*. [13] utilized the separation power of capillary zone electrophoresis for the determination of pralidoxime in human urine. CZE of pralidoxime excreted in the urine reached recovery over 95%, and the results were comparable to those of HPLC performed by Grasshoff *et al*. [26] (for obidoxime) and Houzé *et al*. [12]. The importance of CZE determination of pralidoxime is underlined by two factors. Firstly, CZE does not require any clean-up, meaning that CZE works with 100% recovery of the pyridinium aldoxime content of the sample. Secondly, pralidoxime is eluted much faster than the bis-pyridinium aldoximes. Early elution causes interferences with endogenous substances, especially from the brain.

Reliable determination of trace levels of pralidoxime in the cerebrospinal fluid is also of basic importance. The CSF occupies a specific brain compartment that does not contain any blood. The hitherto used sophisticated technique of

Table 1. Pralidoxime Level Measured in the Serum, Cerebrospinal Fluid (CSF) and Brain in Relation of the Injected (i.m.) Dose. The Ratio of Pralidoxime Level in CSF/Serum and Brain/Serum

Pralidoxime Dose/Rat	Pralidoxime Level in Serum* (μg/mL)	Pralidoxime Level in CSF** (μg/mL)	Pralidoxime Level in Brain* (wet, μg/g)	CSF/Serum (%)	Brain/Serum (%)
100 μM	187.1	3.237	3.149	1.73	1.68
SD	30.37	2.371	0.493		
50 μM	68.5	1.478	1.170	2.16	1.71
SD	13.97	0.479	0.337		
20 μM	23.0	0.663	0.903	2.88	3.92
SD	2.40		0.093		
10 μM	10.0	0.758	0.826	7.58	8.26
SD	2.27		0.291		
1 μM	1.43	0.426	0.566	29.79	39.58
SD	0.26		0.203		

*: Averages of five determinations.

**: Averages of four determination (100μM, 50μM); three determinations (20 μM) and two determinations (10μM and 1 μM).

Sakurada *et al*. [14,19] works with microdialysis around the left striatum, and analysis using HPLC-MS/MS. It can determine pralidoxime after collection of dialysate for a long time period (e.g. 1-h intervals). The time-course of pralidoxime in the rat serum, brain and CSF shows fast onset and offset. Our experimental method (collecting the serum, brain and CSF samples at the same time and analyzing them using CZE) makes a minute to minute comparison of these levels possible.

The present study is the first report of pralidoxime levels in the CSF. In addition, it demonstrates that entry of pralidoxime into the brain, and possibly also into the CSF, is relatively better, when only small doses are administered. This may be related to the specific characteristics of the blood-CSF barrier, the CSF-brain interface and the blood-brain barrier, as discussed by Lorke *et al*. [16]. The use of CZE allows to simultaneously and precisely monitor the time-course of pralidoxime concentrations in the three compartments of the central nervous system and thus offers the possibility to characterize the passage of pralidoxime across these three biological interfaces.

There is a large series of bis-pyridinium aldoximes developed by Kuca *et al*. [2,27,28]. HPLC-UV analysis of K-27 and K-48 was routinely done from rat serum (or from rat plasma). Their level in the rat brain can only be analyzed by sophisticated methods, such as HPLC-electrochemical detector (HPLC-ECD), HPLC-MS or HPLC-MS/MS. The publication of Houzé *et al.* [13] opened the way for pralidoxime analysis in human urine. The sample volume of urine required was relatively large (several hundred milliliters). Our work has successfully determined pralidoxime concentrations also from samples with a very limited amount, such as 10 to 100 μL in the case of CSF.

Our experimental results thus indicated that the pralidoxime level in the serum, brain and CSF can be reliably determined using CZE.

ACKNOWLEDGEMENTS

Skilful technical assistance of Mr. S.M. Nurulain, Mrs. Györgyi Guth and Mr. A. Simon is appreciated. The research was financially supported by the grant of T049492 of OTKA (Hungarian National Granting Agency, Budapest, Hungary).

REFERENCES

[1] Jeyaratnam, J. Acute pesticide poisoning: a major global health problem. *World Health Stat. Quart.,* **1990**, *43*, 139-144.

[2] Kuca, K.; Bielavsky, J.; Cabal, J.; Bielavska, M. Synthesis of a new reactivator of tabun-inhibited acetylcholinesterase. *Tetrahedron Lett.*, **2003**, *44*, 3123.

[3] Suzuki, T.; Morita, H.; Ono, K.; Maekawa, K.; Nagai, R.; Yazaki, Y. Sarin poisoning in Tokyo subway. *Lancet*, **1995**, *345*, 980-981.

[4] Wilson, I.B.; Ginsburg, S. A powerful reactivator of alkylphosphate-inhibited acetylcholinesterase. *Biochim. Biophys. Acta*, **1955**, *18*, 168-170.

[5] Jokanovic, M.; Stojiljkovic, M.P. Current understanding of the application of pyridinium oximes as cholinesterase reactivators in treatment of organophosphate poisoning. *Eur. J. Pharmacol.,* **2006**, *553*, 10-17.

[6] Petroianu, G.A.; Kalász, H. Comparison of the ability of pyridinium aldoximes to reactivate human RBC cholinesterases inhibited by ethyl- and methyl-paraoxon. *Curr. Org. Chem*., **2007**, *11*, 1624-1643.

[7] Tekes, K.; Hasan, M.Y.; Sheen, R.; Kuca, K.; Petroianu, G.; Ludányi, K.; Kalász, H. High-performance liquid chromatographic determination of the plasma concentration of K-27, a novel oxime-type cholinesterase reactivator. *J. Chromatogr. A*, **2006**, *1122*, 84-87.

[8] Kalász, H.; Hasan, M.Y.; Sheen, R.; Kuca, K.; Petroianu, G.; Ludányi, K.; Gergely, A.; Tekes, K. HPLC analysis of K-48 concentration in plasma. *Anal. Bioanal. Chem*., **2006**, *385*, 1062-1067.

[9] Singh, H.; Moorad-Doctor, D.; Ratcliff, R.H.; Wachtel, K.; Castillo, A.; Garcia, G.A. A rapid cation-exchange HPLC method for detection and quantification of pyridinium oximes in plasma and tissue. *J. Anal. Toxicol.*, **2007**, *31*, 69-74.

[10] Utley, D. Determination of 2-hydroxyiminomethyl-1-methyl-pyridinium methanesulphonate (pralidoxime mesylate, P2S) and its degradation products in solution by liquid chromatography *J. Chromatogr*., **1983**, *265*, 311-322.

[11] Gyenge, M.; Kalász, H.; Petroianu, G.A.; Laufer, R.; Kuca, K.; Tekes, K. Measurement of K-27, an oxime-type cholinesterase reactivator by high-performance liquid chromatography with electrochemical detection from different biological samples. *J. Chromatogr. A*, **2007**, *1161*, 146-151.

[12] Houzé, P.; Borron, S.W.; Scherninski, F.; Bousquet, B.; Gourmel, B.; Baud, F.J. Measurement of serum pralidoxime methylsulfate (Contrathion®) by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr. B,* **2005**, *814*, 149-154.

[13] Houzé, P.; Thabet, H.; Delfour, A.; Larrouy, L.; Le Bricon, T.; Baud, F.J. Quantification of pralidoxime methylsulfate (Contrathion®) in human urine by capillary zone electrophoresis *J. Chromatogr. B*, **2005**, *826*, 63-68.

[14] Okuno, S.; Sakurada, K.; Ohta, H.; Ikegaya, H.; Kazui, Y.; Akutso, T.; Takatori, T.; Iwadate, K. Blood-brain barrier penetration of novel pyridine aldoxime methiodide (PAM)-type oximes examined by brain microdialysis with LC-MS/MS. *Toxicol. Appl. Pharmacol*., **2008**, *227*, 8-15.

[15] Csermely, T.; Kalász, H.; Petroianu, G.A.; Kuca, K.; Darvas, F.; Ludányi, K.; Mudhafar, A.A.; Tekes, K. Analysis of pyridinium aldoximes, a chromatographic approach, *Curr. Med. Chem.,* in press.

[16] Lorke, D.E.; Kalász, H.; Petroianu, G.A.; Tekes, K. Entry of oximes into the brain: a review. *Curr. Med. Chem*., **2008**, *15*, 743-753.

[17] Csermely, T.; Petroianu, G.; Kuca, K.; Fürész, J.; Darvas, F.; Gulyás, Z.; Laufer, R.; Kalász, H. TLC of quaternary pyridinium aldoximes, antidotes of organophosphorous esterase inhibitors. *JPC-J. Planar Chromatogr.*, **2007**, *20*, 30-42.

[18] Laufer, R.; Báthori, M.; Csermely, T.; Petroianu, G.; Kuca, K.; Tóth, N.; Kalász, H. TLC determination of hydrophilicity parameter of some pyridinium aldoximes. *J. Liq. Chromatogr.,* **2007***, 30*, 2337-2344.

[19] Sakurada, K.; Matsubara, K.; Shimizu, K.; Shiono, H.; Seto, Y.; Tsuge, K.; Yoshino, M.; Sakai, I.; Mukoyama, H.; Takatori, T. Pralidoxime iodide (2-pAM) penetrates across the blood-brain barrier. *Neurochem. Res*., **2003**, *28,* 1401-1407.

[20] Tekes, K.; Hantos, M.; Gyenge, M.; Csaba, G. Perinatal alcohol exposure enhances nocistatin levels in adulthood. *Addict Biol.*, **2007**, *12,* 173-175.

[21] Szökő, É.; Tábi, T.; Halász, A.S.; Pálfi, M.; Kalász, H.; Magyar, K. Chiral characterization and quantification of deprenyl-N-oxide and other deprenyl metabolites in rat urine by capillary electrophoresis. *Chromatographia* **2004**, *60*, S245-S251.

[22] Szökő, É.; Tábi, T.; Halász, A.S.; Pálfi, M.; Magyar, K. High sensitivity analysis of nitrite and nitrate in biological samples by capillary zone electrophoresis with transient isotachophoretic sample stacking. *J. Chromatogr. A,* **2004**, *1051*, 177-183.

[23] Foret, F., Szökő, É., Karger, B. Trace analysis of proteins by capillary zone electrophoresis with on-column transient isotachophoretic preconcentration. *Electrophoresis,* **1993**, *14*, 417-428.

[24] Guttman, A. Sample stacking during membrane-mediated loading in automated DNA sequencing. *Anal. Chem*., **1999**, *71*, 3598-3602.

[25] Ronai, Z.; Barta, C.; Sasvari-Szekely, M.; Guttman, A. DNA analysis on electrophoretic microchips: effect of operational variables. *Electrophoresis*, **2001**, *22*, 294-299.

[26] Grasshoff, C.; Thiermann, H.; Gillessen, T.; Zilker, T.; Szinicz, L. Internal standard high-performance liquid chromatography method for the determination of obidoxime in urine of organophosphate-poisoned patients. *J. Chromatogr. B Biomed. Sci. Appl.*, **2001**, *753*, 203-208.

[27] Musilek, K.; Jun, D.; Cabal, J.; Kassa, J.; Gunn-More, F.; Kuca, K. Design of a potent reactivator of tabun-inhibited acetylcholinesterase--synthesis and evaluation of (E)-1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (K203). *J. Med. Chem.*, **2007**, *50*, 5514-5518.

[28] Kuca, K.; Bielavsky, J.; Cabal, J.; Kassa, J. Synthesis of a new reactivator of tabun-inhibited acetylcholinesterase. *J. Bioorg. Med. Chem. Lett*., **2003**, *13*, 3545.

Received: 18 November, 2008 Revised: 24 February, 2009 Accepted: 25 February, 2009