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ORIGINAL ARTICLE

In vitro screening of blood-brain barrier penetration of clinically used acetylcholinesterase reactivators

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Summary
In this *in vitro* study, using the HPLC method, we determined the ability of acetylcholinesterase (AChE) reactivators, used clinically, to penetrate the blood-brain barrier (BBB). We evaluated pralidoxime, HI-6, obidoxime, trimedoxime and methoxime – reactivators varying in the position of the oxime group on the pyridinium ring and linker connecting the pyridinium rings. Our results indicated that pralidoxime, a monoquaternary AChE reactivator, was the oxime with the most penetration. Molecular weight seems to be the most important factor for passive transport through the BBB. From the structural perspective, the connecting linker also plays a key role in the ability of the reactivators to penetrate the CNS. In this case, the simple and short linker is favorable for permeation of these compounds. The location of the oxime group on the pyridine ring may also influence passive transport into the brain; the best position of the oxime group seems to be position four.

Key words: blood-brain barrier; CNS penetration; HI-6; obidoxime; HPLC; oxime

INTRODUCTION

The basis of the current standard treatment of organophosphate (OP) poisoning is the administration of cholinesterase reactivators (Eyer 2003, Musílek et al. 2007). These include standard oximes with a similar basic structure but differing in the number of pyridinium rings, in the position of the oxime group on the pyridinium ring and in the linker connecting the pyridinium rings (Kuča et al. 2006). Some mono- and also bisquaternary pyridinium oximes are more or less frequently used in clinical practice. Pralidoxime, obidoxime, trimedoxime, methoxime and HI-6 are typical members of this family (Kuča et al. 2007). The mechanism of their action is hydrolytical cleaving of the OP from acetylcholinesterase (AChE; 3.1.1.7), restoring its enzymatic function. This reactivation of the inhibited enzyme is dependent on the type of OP and, on the reactivator used (Bajgar 2004, Žďárová Karasová et al. 2009).

Reactivation of AChE in the peripheral and also in the central nervous system (CNS) is very important for the survival of an organism poisoned with OP. The question of their penetration through the blood brain barrier (BBB) as well as the possibility of their achievement of effective brain concentration is under discussion (Bajgar et al. 2007a).

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There is direct and indirect evidence for the ability of oximes to penetrate the BBB. The indirect evidence is based on AChE reactivation in the brain following OP intoxication (Bajgar et al. 1972, Kassa et al. 2007, Žďárová Karasová et al. 2008). The direct evidence for presence of oximes in the brain has been demonstrated by Sakurada et al. (2003) using microdialysis detection of pralidoxime. Similar observations have been described by other authors (Falb and Erdmann 1969, Cassel et al. 1997, Lorke et al. 2007, Petroianu et al. 2007).

The main aim of this study is to predict the extent of BBB penetration by standard AChE reactivators. Immobilized artificial membrane (IAM) chromatography was utilized for the assessment of these pharmacokinetic properties of the different oximes (Yoon et al. 2006). The method was validated on a set of 21 structurally varying therapeutics and subsequently applied to clinically used monoquaternary (pralidoxime) and bisquaternaly AChE reactivators (obidoxime, trimedoxime, HI-6 and methoxime).

MATERIALS AND METHODS

Chemicals

Atenolol, β-estradiol, caffeine, cefuroxime, chlorpromazine, cimetidine, corticosterone, desipramine, enalapril, hydrocortisone, ibuprofen, imipramine, lomefloxacin, loperamide, nadolol, piroxicam, progesterone, promazine, propranolol, and testosterone were purchased from Sigma Aldrich (Steinheim, Germany). Acetonitrile gradient grade LiChrosolv was purchased from Merck (Darmstadt, Germany). KH_2PO_4, Na_2HPO_4, KCl, and NaCl were purchased from Lachema (Neratovice, The Czech Republic). AChE reactivators were synthesized earlier in our laboratory (Musílek et al. 2006, Kuča et al. 2008). Water was reverse osmosis pure.

Apparatus

The HPLC system consisted of a P200 gradient pump (Spectra-Physics Analytical, Fremont, USA), a 7125 injection valve – 10 µl loop (Rheodyne, Cotati, USA), an UV1000 detector (Spectra-Physics Analytical, Fremont, USA) and a CSW Chromatography Station 1.5 software (DataApex, Praha, Czech Republic).

Chromatographic condition

Conditions for prediction (analysis)

An IAM.PC.DD 2 (150 × 4.6 mm; 12 µm) column (Regis Technologies, Morton Grove, USA) was used for analysis. The mobile phase was 80% PBS and 20% acetonitrile (v/v) with pH adjusted to 5.5 and 7.0 using Na_2HPO_4. The phosphate-buffered saline (PBS) was prepared with 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, and 8.1 mM Na_2HPO_4. It was delivered isocratically at a flow-rate of 1 ml/min. The absorbance was measured at 210 nm. All chromatograms were obtained at 37 °C.

Conditions for samples

For the analyses a 125 × 3 mm I.D. Purospher RP-18e (5 µm) column (Merck, Darmstadt, Germany) was used. The mobile phase was 24% acetonitrile and 76% water (v/v), containing 5 mM octane-1-sulfonic acid sodium salt, 5 mM tetramethylammonium chloride. It was delivered isocratically at a flow-rate of 1 ml/min. The absorbance was measured at UV_{max} of each reactivator. All chromatograms were obtained at 24 °C.

RESULTS

The most important coefficient for the determination of IAM partition is k_{IAM} (IAM capacity factor), which was calculated as

$$\mathbf{k_{IAM} = (t_r - t_0)/t_0}$$

where $\mathbf{t_r}$ is the retention time of the drug and $\mathbf{t_0}$ is the hold up time of the column.

In this study, the k_{IAM} was determined for twenty-one reference drugs. The k_{IAM} values were determined with a mobile phase of pH 7.4, although Yoon has recommended using a mobile phase of pH 5.5 because it provided better results. Our experiment was, however, carried out with a mobile phase of a higher pH because of the need to establish an environment similar to that in human body. This change of pH range may haved markedly changed the state of the chemical ionization of the drugs. Chemical ionization is a very important factor which may in turn significantly change the possibility of molecule penetrating through the BBB.

According to Yoon et al. (2006) the assortment of drugs which can cross the BBB (CNS+) and those which do not penetrate into the brain (CNS–) was chosen based on k_{IAM} corrected by the molecular weight (M_W). The assortment of pH 7.4 was most successful with the power function of the molecular weight set at 4 (Yoon et al. 2006). The designated formula was:

$$\mathbf{X = k_{IAM}/MW^4 \times 10^{10}} \quad .$$

In addition a calculation was made of the predicted constants of the synthesized compounds – the partition coefficient (LogP), the molecular polar surface area (PSA) and the molecular weight (MW). In respect of LogP, it can be clearly seen that all substances are more soluble in water than in octanol. Fig. 1 illustrates the correlation between log P and log P and k_{IAM}/MW^4, the correlation coefficient (r^2) being 0.6677 at pH 7.4.

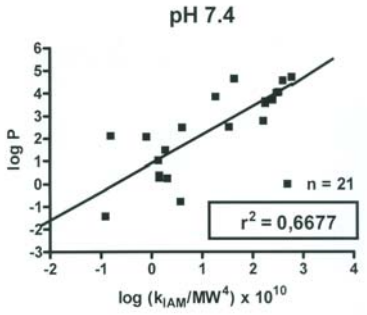


Fig. 1. **Correlation between log P and k_{IAM}/MW^4 determined at the mobile phase pH of 7.4.**

The PSA (the sum of surfaces of polar atoms: oxygens, nitrogens and attached hydrogens, in a molecule) is a parameter very useful for the prediction of drug transport properties (Zhu et al. 2002). The PSA has been previously shown to correlate with human intestinal absorption (Palm et al. 1998, Clark 1999). When PSA is applied to a larger and more diverse compound set, however, outliers become more frequent (Zhu et al. 2002). In this study, a good correlation was observed between PSA and k_{IAM}/MW^4 with a correlation of 0.7199 at the mobile phase of pH of 7.4 (Fig. 2).

The CNS-drugs showed evident inability to bind to the phosphatidylcholine column and have X values less than 0.50, whereas the CNS+ drugs bound much better and their X values were distinctively higher than 1.00.

The utility of the optimized prediction method was examined for five reactivators of AChE, commonly used in therapy, which differ in their chemical structure (Table 1). At Fig. 3 is a HPLC chromatogram of the commonly used reactivators (pralidoxime, obidoxime, trimedoxime, HI-6, methoxime) with different retention times.

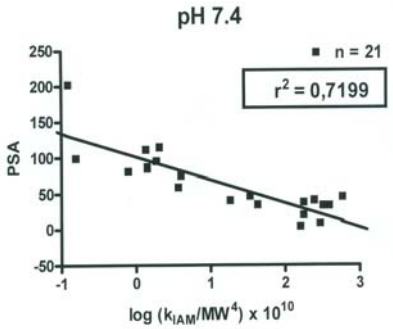


Fig. 2. **Correlation between polar surface area (PSA) and k_{IAM}/MW^4 determined at the mobile phase pH of 7.4.**

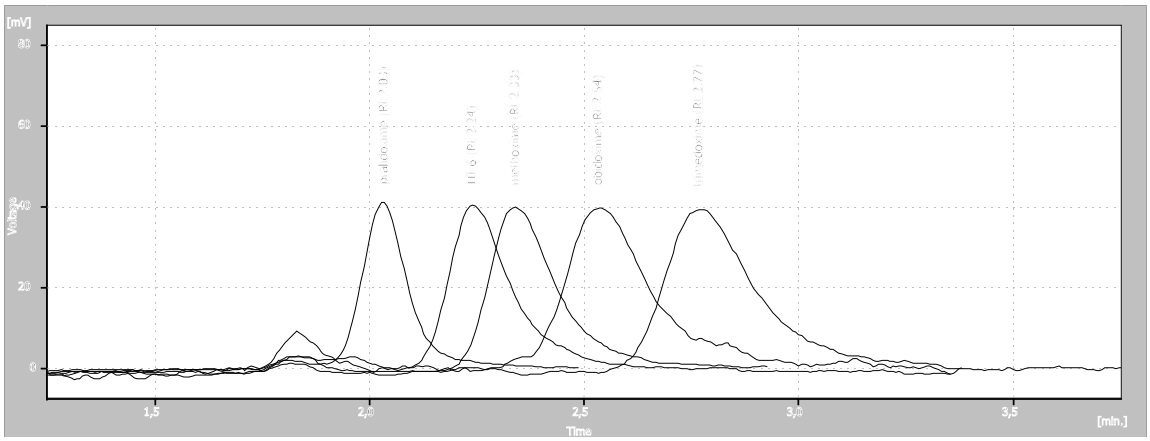
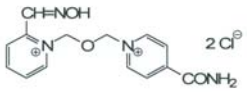
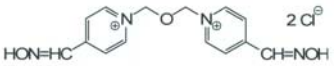
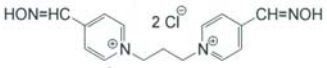
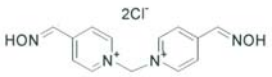
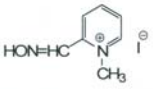


Fig. 3. **HPLC chromatogram of the clinically used reactivators (pralidoxime, obidoxime, trimedoxime, HI-6, methoxime) with retention times.**

Table 1. **The group of tested reactivators, their results.**

Term	Formula	MW	Rt	Result (X)	pKa	log P	PSA
HI-6		288.30	2.24	**1.047**	3.87	-5.97	85.00
obidoxime		288.30	2.54	**1.381**	8.28	-6.09	82.17
trimedoxime		286.33	2.77	**1.682**	9.40	-6.61	72.94
methoxime		258.28	2.33	**1.780**	8.23	-6.30	72.94
pralidoxime		137.16	2.03	**15.886**	5.58	-3.12	39.3

DISCUSSION

There are many questions still to be answered which are focused on the presence of reactivators in the brain. It has generally been accepted that oximes as quaternary compounds are not able to penetrate the BBB (Kassa et al. 2008). As already written above, there is much direct and indirect evidence for the ability of oximes to pass through into the brain (Falb and Erdmann 1969, Cassel et al. 1997, Lorke et al. 2007, Petroianu et al. 2007).

The AChE reactivation is very important in CNS, because there are so many changes in the physiology of the brain after OP intoxication (Kuča and Kassa 2004). They cause a strong cholinesterase inhibition with subsequent changes in the level of neurotransmitters including acetylcholine and catecholamines (Bajgar et al. 2007b). Also recorded in CNS were changes in membrane permeability, in the influence of BBB permeability and in metabolic imbalances (changes in the brain energy metabolism during soman intoxication, influence of the oxidative metabolism and ATP level in the brain) (Gupta 2004).

The pontomedullar area, where respiration is regulated (controlled by cholinergic neurons) is of particular importance. Depression of the central respiratory control centres in the pontomedullar area is considered as a primary event leading to death (Goswany et al. 1994, Sungur and Guven 2001, Kubin and Fenik 2004). When the AChE reactivation is present in this area, a good therapeutic effect is observed. The survival of intoxicated animals is correlated with AChE activity in the pontomedullar area (Bajgar et al. 2007b).

According to our hypothesis, the results obtained in our study proved a dependency between CNS penetration and the structure of various reactivators. The less penetrative bisquaternary compounds were HI-6 and obidoxime. Oxime HI-6, is a very promising antidote against the broad spectrum of OP (Kuča et al. 2009). Obidoxime is effective against tabun and pesticide intoxications. There are three differences in the chemistry of these clinically used oximes. The position of the oxime group on the pyridinium ring is the first (obidoxime – 4, HI-6 – 2 position). Then, obidoxime has two oxime groups of HI-6 instead of one. Finally, the second obidoxime oxime group is replaced by the carbamoyl group in the HI-6 structure.

If these results are compared with those of trimedoxime, it can clearly be seen that the difference in the connecting linker also influences BBB penetration. The oxygen in the linker between two pyridinium rings is not conducive to BBB permeation. The best bisquaternary structure from our point of view is methoxime which has two oxime groups in the position four on the pyridinium rings and very short linker without oxygen.

It is known that the most important parameters of influence in BBB passive penetration are MW and the presence of sufficient liposolubility (Bellawance et al.

2008). This was confirmed also in our study. The oxime with lowest MW (monoquaternary AChE reactivator, pralidoxime) was the best penetrating structure (Žďárová Karasová et al. 2010). Our results were indirectly confirmed in other *in vivo* studies, (Lorke et al. 2007, 2008, Petroianu et al. 2007, Kalasz et al. 2009). The monoquaternary oximes penetrated 10 times more than bisquaternary compounds.

On the basis of our results we can predict the more permeating oxime structures. This knowledge may be useful in the synthesis of more effective AChE reactivators.

CONCLUSION

In conclusion, we have tested an HPLC method with UV detection for the prediction of five clinically used AChE reactivators. According to our results molecular weight seems to be the most important factor for passive transport throught the BBB. Secondly, based on results obtained, even small changes in the chemical structure of oxime (connecting linker, location of oxime group on the pyridinium ring and also substitution of oxime group) are important in influencing the extent of brain penetration.

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REFERENCES

Bajgar J: Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. Adv Clin Chem 38:151–216, 2004.

Bajgar J, Jakl A, Hrdina V: The influence of obidoxime on acetylcholinesterase activity in different parts of the mouse brain following isopropylmethyl phosphonofluoridate intoxication. Eur J Pharmacol 19:199–202, 1972.

Bajgar J, Fusek J, Kuča K, Bartošová L, Jun D: Treatment of organophosphate intoxication using cholinesterase reactivators: facts and fiction. Mini Rev Med Chem 7:461–466, 2007a.

Bajgar J, Kuča K, Fusek J, Karasová J, Kassa J, Cabal J, Bláha V: Inhibition of blood cholinesterases following intoxication with VX and its derivatives. J Appl Toxicol 27:458–463, 2007b.

Bellawance MA, Blanchette M, Fortin D: Recent advances in blood-brain barrier disruption as a CNS delivery strategy. AAPS J 10:166–177, 2008.

Cassel G, Karlsson L, Waara L, Ang KW, Goransson-Nyberg A: Pharmacokinetics and effects of HI 6 in blood and brain of soman-intoxicated rats: a microdialysis study. Eur J Pharmacol 30:43–52, 1997.

Clark DE: Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena: Prediction of intestinal absorption potential. J Pharm Sci 88:807–814, 1999.

Eyer P: The role of oximes in the management of organophosphorus pesticide poisoning. Toxicol Rev 22:165–190, 2003.

Falb A, Erdmann WD: Penetration of 14C-obidoxime through the so-called blood-brain barrier of mice and rats. Arch Toxicol 24:123–132, 1969.

Goswany R, Chaudhuri A, Mahashur AA: Study of respiratory failure in organophosphate and carbamate poisoning. Heart Lung 23:466–472, 1994.

Gupta RC: Brain regional heterogeneity and toxicological mechanisms of organophosphates and carbamates. Toxicol Mech Methods 14:103–143, 2004.

Kalász H, Szöko E, Tábi T, Petroianu GA, Lorke DE, Omar A, Alafifi S, Jasem A, Tekes K: Analysis of pralidoxime in serum, brain and CSF of rats. Med Chem 5:237–241, 2009.

Kassa J, Karasová J, Vašina L: The evaluation of neuroprotective efficacy of newly developed oximes (K 074, K 075) and currently available oximes (obidoxime, HI-6) in cyclosarin-poisoned rats. J Appl Toxicol 27:621–630, 2007.

Kassa J, Jun D, Karasová J, Bajgar J, Kuča K: A comparison of reactivating efficacy of newly developed oximes (K074, K075) and currently available oximes (obidoxime, HI-6) in soman, cyclosarin and tabun-poisoned rats. Chem Biol Interact 175:425–427, 2008.

Kubin L, Fenik V: Pontine cholinergic mechanisms and their impact on respiratory regulation. Respir Physiol Neurobiol 143:235–249, 2004.

Kuča K, Kassa J: Oximes-induced reactivation of rat brain acetylcholinesterase inhibited by VX agent. Hum Exp Toxicol 23:167–171, 2004.

Kuča K, Jun D, Musílek K: Structural requirements for acetylcholinesterase reactivators. Mini Rev Med Chem 6:269–277, 2006.

Kuča K, Jun D, Bajgar J: Currently used cholinesterase reactivators against nerve agent intoxication: Comparison of their effectivity *in vitro*. Drug Chem Toxicol 30:31–40, 2007.

Kuča K, Stodůlka P, Hrabinová M, Hanušová P, Jun D, Doležal B: Preparation of oxime HI-6 (dichloride and dimethanesulphonate)-antidote against nerve agents. Def Sci J 58:399–404, 2008.

Kuča K, Musílek K, Jun D, Pohanka M, Žďárová Karasová J, Novotný L, Musilová L: Could oxime HI-6 really be considered as "broad-spectrum" antidote? J Appl Biomed 7:143–149, 2009.

Lorke DE, Hasan MY, Nurulain SM, Sheen R, Kuča K, Petroianu GA: Entry of two new asymmetric bispiridium oximes (K-27 and K-48) into the rat brain: comparison with obidoxime. J Appl Toxicol 27:482–490, 2007.

Lorke DE, Kalasz K, Petroianu GA, Tekesz K: Entry of oximes into the brain: A review. Curr Med Chem 15:743–753, 2008.

Musílek K, Lipka L, Račáková V, Kuča K, Jun D, Dohnal V, Doležal M: New methods in synthesis of acetylcholinesterase reactivators and evaluation of their potency to reactivate cyclosarin-inhibited AChE. Chem Pap 60:48–51, 2006.

Musílek K, Kuča K, Jun D, Doležal M: *In vitro* reactivation potency of bispyridinium (E)-but-2-ene linked acetylcholinesterase reactivators against tabun-inhibited acetyl-cholinesterase. J Appl Biomed 5:25–30, 2007.

Palm K, Luthman K, Ungell AL, Strandlung G, Beigi F, Lundahl P: Evaluation of dynamic polar molecular surface area as predictor of drug absorption: comparison with other computational and experimental predictors. J Med Chem 41:5382–5392, 1998.

Petroianu GA, Lorke DE, Hasan YM, Adem A, Sheen R, Nurulain SM, Kalasz H: Paraoxon has only a minimal effect on pralidoxime brain concentration in rats. J Appl Toxicol 27:350–357, 2007.

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 28:1401–1407, 2003.

Sungur M, Guven M: Intensive care management of organophosphate insecticide poisoning. Crit Care 5:211–215, 2001.

Yoon HC, Kim SJ, Shin BS, Lee KC, Yoo SD: Rapid screening of blood-brain barrier penetration of drugs using the immobilized artificial memebrane phosphatidylchline column chromatography. J Biomol Screen 11:13–20, 2006.

Žďárová Karasová J, Kassa J, Jung Y-S, Musílek K, Pohanka M, Kuča K: Effect of several new and currently available oxime cholinesterase reactivators on tabun-intoxicated rats. Int J Mol Sci 9:2243–2252, 2008.

Žďárová Karasová J, Bajgar J, Novotný L, Kuča K: Is a high dose of Huperzine a really suitable for pretreatment against high doses of soman? J Appl Biomed 7:93–99, 2009.

Žďárová Karasová J, Stodůlka P, Pohanka M, Kuča K (2010): *In vitro* screening of blood-brain barrier penetration of monoquaternary acetylcholinesterase reactivators. Anal Lett – In press. DOI 10.1080/00032710903502082.

Zhu C, Jiang L, Chen TM, Hwang KK: A comparative study of artificial membrane permeability assay for high throughput profiling of drug absorption potential. Eur J Med Chem 37:399–407, 2002.