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Partition of bispyridinium oximes (trimedoxime and K074) administered in therapeutic doses into different parts of the rat brain

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ABSTRACT

The penetration of acetylcholinesterase reactivators (oximes) into the central nervous system is typically restricted by the blood–brain barrier. Although oximes are highly hydrophilic compounds, some contradictory results confirming permeation into the brain exist. The aim of this study is to verify the penetration of oximes through the blood–brain barrier and to detect their levels achieved in different brain regions 60 min after the administration.

It was confirmed that oximes are able to penetrate into the brain after injection of therapeutic doses corresponding with 5% of LD_{50}. The level in whole brain was 0.58% for trimedoxime and 0.85% for the experimental drug oxime K074 as the percentage of their plasma concentration. The highest concentration was found in frontal cortex (trimedoxime 2.27%; oxime K074 0.95%) and lowest in basal ganglia (trimedoxime 0.86%; oxime K074 0.42%).

Entry of oximes into the brain is minimal, but some low reactivation effect should be expected. The reactivation potency of oximes might be higher or lower, depending on the real oxime concentration in a given area.

1. Introduction

The blood–brain barrier (BBB) plays a vital role in the protection of the central nervous system (CNS). Only 5% of known xenobiotics are able to penetrate the BBB and induce an action in the brain tissue [1]. The BBB is also important in maintaining homeostasis of the CNS that is essential for its normal functionality. The environment of BBB is lipophilic so it is virtually impossible for hydrophilic xenobiotics to penetrate into the CNS. Unfortunately, acetylcholinesterase (AChE; 3.1.1.7) reactivators, very potent substances in the treatment of organophosphorus poisoning have low lipophilic properties [2].

Several *in vitro* and *in vivo* approaches are presently used in BBB penetration studies. The BBB penetration process, as studied by *in vitro* methods, is characterised by several molecular descriptors characterising the studied compounds. The octanol–water partition coefficient ($\log P$) is one of the molecular descriptors widely used for the determination of lipophilic/hydrophilic properties [3,4]. Oxime-type AChE reactivators such as the pyridinium monoquaternary or bisquaternary compounds have negative $\log P$ (low lipophilic character), resulting in low BBB penetration [5]. Polar surface area (PSA) [6], number of rotatable bonds [7], number of H-bond donors and number of H-bond acceptors represent more molecular descriptors available for the evaluation of BBB permeability and can also suggest possible peripheral compartment distribution within the CNS [8–11].

The first *in vivo* studies of oximes were conducted with paraoxon. It was injected into the animal brain and changes of AChE activity in the CNS were evaluated [12]. Minor elevation of AChE activity in the tested animal brains was interpreted as reactivation of AChE by paraoxon [13,14]. However, simply measuring AChE activity in the brain does not provide sufficient evidence of BBB penetration by the oxime. The main reason for this is that the residue concentration of the oxime in brain vessels can affect reactivation process of AChE in the actual brain tissue. Oxime brain tissue contamination can easily occur during the sample homogenisation. Other methods such as radioactive tracer techniques (^{14}C labelled oximes) and high-performance liquid chromatography (HPLC) have been developed to study oxime CNS penetration. Also, these methods are useful to determine the concentration of the penetrated oxime. The HPLC method seems to have the most relevant measurements of real oxime concentration in the brain tissue

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Table 1
The intra-day and inter-day precision of trimedoxime and oxime K074.

Concentration	Plasma			Concentration	Brain		
Added (μg/ml) of trimedoxime	C.V. (%)	Mean ± standard deviation	*n*	Added (ng/ml) of trimedoxime	C.V. (%)	Mean ± standard deviation	*n*
Inter-day				Inter-day			
5.0	4.04	4.9 ± 0.1	10	20.0	3.54	19.6 ± 0.5	10
10.0	3.21	9.8 ± 0.4	10	40.0	3.41	40.3 ± 0.6	10
20.0	3.98	20.8 ± 0.9	10	80.0	4.09	80.4 ± 1.0	10
30.0	2.81	30.1 ± 1.1	10	160.0	3.24	161.3 ± 2.2	10
40.0	3.11	39.6 ± 1.4	10	320.0	4.61	318.1 ± 3.1	10
Intra-day				Intra-day			
5.0	4.68	5.1 ± 0.5	3	20.0	5.11	19.7 ± 0.7	3
10.0	3.33	9.8 ± 0.6	3	40.0	4.89	39.5 ± 0.8	3
20.0	3.95	19.7 ± 1.1	3	80.0	4.35	78.9 ± 1.3	3
30.0	3.57	30.2 ± 0.9	3	160.0	4.82	159.3 ± 1.4	3
40.0	3.24	39.9 ± 1.1	3	320.0	3.99	321.1 ± 3.2	3
Added (μg/ml) of oxime K074				Added (ng/ml) of oxime K074			
Inter-day				inter-day			
5.0	3.29	5.2 ± 0.3	10	20.0	3.62	19.8 ± 0.4	10
10.0	3.16	10.3 ± 0.5	10	40.0	3.55	39.4 ± 0.5	10
20.0	5.22	19.8 ± 0.9	10	80.0	3.76	80.2 ± 1.1	10
30.0	5.07	29.5 ± 1.2	10	160.0	3.22	160.3 ± 1.3	10
40.0	3.47	39.3 ± 1.1	10	320.0	4.05	321.1 ± 1.7	10
Intra-day				Intra-day			
5.0	4.04	4.9 ± 0.2	3	20.0	4.92	19.9 ± 0.3	3
10.0	3.31	10.0 ± 0.5	3	40.0	5.07	39.6 ± 0.7	3
20.0	4.77	19.4 ± 1.2	3	80.0	4.23	79.5 ± 0.8	3
30.0	3.01	29.7 ± 1.1	3	160.0	5.03	158.9 ± 2.2	3
40.0	4.56	39.7 ± 1.7	3	320.0	4.27	320.1 ± 2.3	3

[2]. Understandably, improvement of the method is closely linked with the development of highly sensitive detectors and new types of columns meaning the limit of oxime detection is continuously improving.

The aim of this study is to verify data of oxime penetrations into different brain sections (whole brain, pontomedullar area, basal ganglia and frontal cortex) after therapeutic dose application. These brain areas were chosen because of different sensitivity to AChE inhibitors [15]. Moreover, the ability to achieve effective oxime concentration in the brain to elicit pharmacological effect is also of great importance. The assumption is that a relatively small quantity of reactivator in some parts of the CNS might be enough to activate the minimum concentration of blocked AChE to save lives of intoxicated patients [16]. As can be seen on animal model, small increase of AChE activity in brain areas correlates with survival/death of intoxicated animals by organophosphate inhibitors [17].

2. Materials and methods

2.1. Chemicals

Oximes (trimedoxime and oxime K074, Fig. 1) were synthesized in laboratory at the Department of Toxicology as proposed by Kuca et al. [31]. Their purity was 99%. Acetonitrile gradient grade LiChrosolv, octane-1-sulfonic acid sodium salt (99%) and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). Trichloroacetic acid (99.5%) was purchased from Sigma–Aldrich (branch in Prague, Czech Republic). The water was double distilled and deionized and of HPLC grade.

2.2. HPLC instrumentation and separation conditions

Plasma samples were analyzed by reversed phase HPLC with UV detection. 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 Clarity 2.6.5.517 software (DataApex, Prague, Czech Republic) was used to analyze the results.

The analytical column was LiChrospher® 60, 250 × 4.6 (5 μm) containing a guard column 4 × 4 (RP-select B) (Merck, Darmstadt, Germany). The mobile phase composition consisted of 24:76 (v/v) acetonitrile and aqueous component containing 5 mmol/l sodium octane sulfonate and 5 mmol/l tetramethylammonium chloride. The pH was adjusted to 2.1 with phosphoric acid. The flow rate of the mobile phase was 1 ml/min [18]. The absorption maximum was 290 nm for trimedoxime and 285 nm for K074. Separation was carried out at 22 °C.

Brain samples were analyzed by using another chromatographic system consisted of an isocratic pump LC-10AD (Shimadzu, Duisburg, Germany), a 7125 injection valve – 10 μl loop (Rheodyne, Cotati, USA), Coulochem II detector with analytical cell model 5011 (ESA, Bedford, USA) and a Clarity software was used to analyze results.

The same type of analytical column and guard column was used. The mobile phase composition consisted of 18:82 (v/v) acetonitrile and aqueous component containing 6 mM for trimedoxime and 7 mM sodium octane sulfonate for oxime K074 analysis. The pH was adjusted to 2.2 for trimedoxime analysis and 2.7 for analysis of oxime K074 by using H_3PO_4. The flow rate of the mobile phase was 1.5 ml/min.

Measurement cells had respective potentials of +350 mV/+650 mV. Potential of the guard cell was +1000 mV. The detector gain was set at 20 nA. All chromatograms were obtained at conditioned room temperature (23 °C).

2.3. Preparation of calibration standards and calculation of assayed concentrations

Calibration standards were established using blank rat plasma spiked with oximes in range (1, 3.12, 6.25, 12.5, 25.0 and 50.0 μg/ml oxime, in triplicates) and brain homogenates were also spiked by oximes in range (10.0, 20.0, 40.0, 80.0, 160.0 and 320.0 ng/ml, in triplicates).

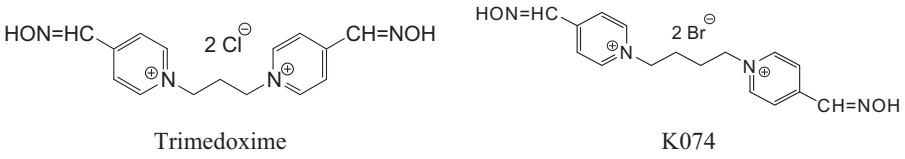

Fig. 1. Structures of tested oximes.

Regression analysis of the relationship between peak areas and theoretical concentrations of calibration standards was performed by the method of least-squares using Prism 4 (Graph Pad Software, USA).

2.4. Precision

Intra-day precision was determined by repeating the measurement of spiked samples ten times with five different concentrations (plasma: 5, 10, 20, 30 and 40 μg/ml; $n = 4$) or (brain homogenate: 20, 40, 80, 160 and 320 ng/ml; $n = 4$) on the same day. Inter-day precision was determined by calculating the means of four replicates of samples spiked by both oximes (plasma: 5, 10, 20, 30 and 40 μg/ml) or (brain homogenate: 20, 40, 80, 160 and 320 ng/ml; $n = 4$) on three subsequent days.

The intra-day and inter-day coefficients of variations (C.V.) of both oximes are summarized in Table 1.

2.5. Accuracy in plasma and brain samples

Trimedoxime in plasma samples (5, 10, 20, 30 and 40 μg/ml) was quantified once a day on ten different days. Accuracy was 98.8% (5 μg/ml); 98.9% (10 μg/ml); 98.5% (20 μg/ml); 99.1% (30 μg/ml) and 98.7% (40 μg/ml), respectively.

Oxime K074 in plasma samples in the same concentrations was quantified by the same way. The accuracy was 98.0% (5 μg/ml); 97.9% (10 μg/ml); 98.0% (20 μg/ml); 98.2% (30 μg/ml) and 98.1% (40 μg/ml).

Trimedoxime in brain samples (20, 40, 80, 160 and 320 ng/ml) was quantified once a day on ten different days. Accuracy was 97.8% (20 ng/ml); 97.7% (40 ng/ml); 98.6% (80 ng/ml); 99.0% (160 ng/ml) and 99.2% (320 ng/ml), respectively.

Oxime K074 in brain samples (20, 40, 80, 160 and 320 ng/ml) was quantified once a day on ten different days. Accuracy was 98.1% (20 ng/ml); 98.3% (40 ng/ml); 98.8% (80 ng/ml); 99.2% (160 ng/ml) and 99.3% (320 ng/ml), respectively.

2.6. Limit of quantification (LOQ) and limit of detection (LOD) in plasma and brain samples

LOQ was calculated as the lowest concentration of samples which were measured with a precision of 20% and relative error of ± 20%. The LOD was derived from LOQ according to the equation LOD = (3.3 × LOQ)/10.

The LOQ (plasma samples) for trimedoxime was 1.20 μg/ml and the LOD 0.40 μg/ml. The LOQ for oxime K074 was 2.80 μg/ml and LOD was 0.92 μg/ml.

The LOQ (brain samples) for trimedoxime was 40.00 ng/ml and the LOD 13.20 ng/ml. The limit of LOQ for oxime K074 was 20.00 ng/ml and LOD was 6.60 ng/ml.

2.7. Animal treatment

Male Wistar rats (body weight 220 ± 10 g; Anlab Inc., Prague, Czech Republic) were kept in the Vivarium of Faculty of Military Health Sciences, Hradec Kralove and temperature (22 ± 2 °C), humidity (55 ± 6%), and 12 h light–dark cycle were regulated. Standard laboratory food and tap water were available *ad libitum*. The experiment was performed under permission and supervision of the Ethic Committee of the Faculty of Military Health Sciences, University of Defence; Hradec Kralove, Czech Republic.

After seven days of rats' acclimatization, the oximes were injected intramuscularly (i.m.; hind limb) with 7.5 mg/kg of body weight of trimedoxime ($N = 6$) and 23.00 mg/kg of oxime K074 ($N = 6$). The injected doses corresponded with therapeutical doses (according to the 5% of LD_{50}). Oximes were freshly dissolved in physiological solution before the applications (0.1 ml/100 g of animal weight).

Animals were narcotized with carbon dioxide 60 min after oxime injection. After that the thoracic cavity was opened. The blood was obtained directly from heart and was collected in potassium EDTA coated tubes, gently mixed and centrifuged at 3000 × *g* for 15 min (Universal 320R, Hettich, Germany) to obtain plasma. The plasma samples were stored at −80 °C until HPLC analysis.

The blood in brain vessels also contained oximes. It is not suitable for measurement of real oximes levels in brain tissue. The animals were perfused transcardially by saline solution (0.9% NaCl) for 10 min (50 ml/min). After perfusion, the skull was opened and the brain was carefully removed. The brains were stored at −80 °C until the HPLC analysis.

Before the HPLC assay from still frozen brains three parts were cut: pontomedullar area, basal ganglia and frontal cortex.

2.8. Sample preparation procedure

Plasma samples (divided into three parts) were mixed with 20% trichloroacetic acid (TCA) to precipitate the proteins (ratio: 1:5, plasma/TCA). After that, the samples were centrifuged at 10,000 rpm for 4 min (M240R centrifuge, Hettich, Germany). The supernatant was removed and directly injected into the HPLC (total volume 10 μl, in triplicates).

Brain tissues (the whole brain and parts of brain: pontomedullar area, basal ganglia and frontal cortex) were pre-homogenized with (DI 25 Homogenizer, IKA-WERKE) for 1 min and subsequently was used a VialTweeter UIS250v (Hielscher – Ultrasound Technology, Germany) in distilled water (ratio 1:4; brain tissue/distilled water) for 5 min. Homogenates were divided into three parts and subsequently were precipitate by using 20% TCA (ratio: 1:4; brain homogenate/TCA). After that, the samples were centrifuged at 14000 *g* for 15 min (M240R centrifuge, Hettich, Germany). The supernatants were extracted and directly injected into the HPLC (total volume 10 μl, in triplicates).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, California). The mean and SD were calculated.

Table 2
Oximes concentrations in plasma and different brain segments – mean ± SD.

		Brain (ng/g)	Plasma (μg/ml)	Plasma partition coefficient
Trimedoxime	Frontal cortex	124.8 ± 16.19		2.57
	Basal ganglia	41.47 ± 15.54	4.84 ± 0.29	0.86
	Pontomedullar area	70.72 ± 59.33		1.46
	Whole brain	28.21 ± 13.47		0.58
K074	Frontal cortex	180.8 ± 37.55		0.95
	Basal ganglia	80.06 ± 26.13		0.42
	Pontomedullar area	171.9 ± 50.28	18.96 ± 0.43	0.91
	Whole brain	160.3 ± 30.97		0.85

Plasma partition coefficient – plasma unbound fraction/brain unbound fraction of measured compounds (%).

3. Results

3.1. Tolerability

All animals tolerated the injection of either trimedoxime or K074 very well. No signs of discomfort, such as pain and convulsion of hind limb muscles, were observed during the 60 min follow-up period.

3.2. HPLC assay for trimedoxime and K074

Endogenous compounds in the plasma separated well from trimedoxime and K074 which eluted at 4.7 and 4.6 min, as shown in the chromatograms. Similarly, trimedoxime and K074 observed a linear relationship ($R^2 > 0.998$) between the peak areas and the concentrations of calibration standards in the range of 1–50 μg/ml.

The separations of oximes in brain from endogenous compounds were also satisfactory. The retention times were 26.0 for trimedoxime and 25.7 min for oxime K074 (Figs. 2 and 3). Linear relationship ($R^2 > 0.963$) was found between the peak areas and the concentrations of trimedoxime and K074 calibration standards in the range of 10–320 ng/ml.

3.3. Penetration of oximes through the BBB

The trimedoxime concentration in plasma was only 4.84 ± 0.29 μg/ml 60 min after the i.m. application. Plasma level of oxime K074 was higher: 18.96 ± 0.43 μg/ml in the same time interval. This discrepancy may be explained by different doses of application. The doses of oximes were equitoxical not equimolar and also some differences in oxime time-course changes plasma concentrations were previously published.

Overview of trimedoxime and oxime K074 levels in whole brain and chosen brain segments are given in Table 2. The measured concentration of trimedoxime in whole brain corresponds to 28.21 ± 13.47 ng/g (per gram wet tissue) and 160.3 ± 30.97 ng/g for oxime K074. It seems that distribution into brain segments of both oximes obey the same rules: the highest concentrations were found in frontal cortex, the lowest in basal ganglia.

4. Discussion

Oxime effects in the whole brain have been intensely and controversially discussed. Due to the structure and calculated physico-chemical descriptors (log *P*, PSA) AChE reactivators are not convenient compounds for BBB penetration [3,10,19,20]. According to the previously published *in vitro* data differences in the ability of penetrating the BBB between monoquaternary and bisquaternary oximes exist. Monoquaternary oximes have up to 10-fold higher potency to penetrate through the BBB than bisquaternary oximes [21]. Penetration ability also depends on the linker between pyridinium rings in bisquaternary oximes and the position of the oxime group on the pyridinium ring. These structural formations can have slightly positive influence on the BBB penetration [22].

Although, these *in vitro* data exist, it is still necessary to compare them with *in vivo* (rodents) studies especially if previously published *in vivo* data are controversial. The first attempt to measure oxime concentration in the CNS was performed in the 1960th with the radioactive tracer technique. The tracer (^{14}C) labelled pralidoxime (monoquaternary) and obidoxime (bisquaternary) were used. The final brain tissue concentrations were calculated against

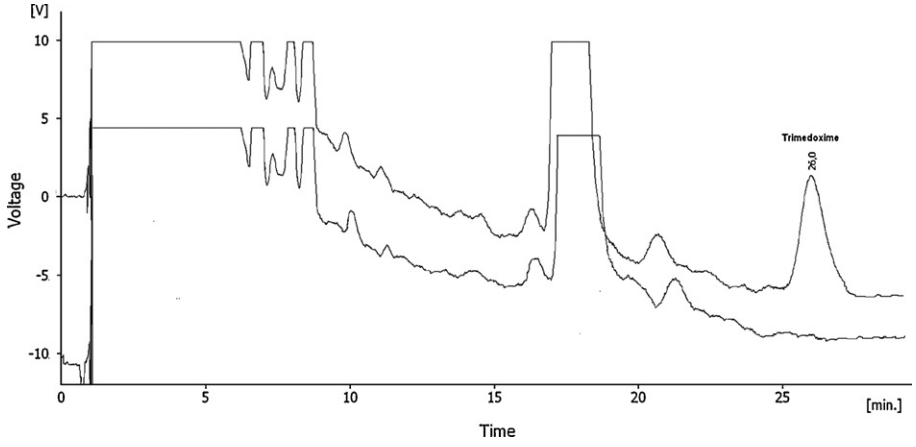

Fig. 2. HPLC chromatogram of trimedoxime in rat brain.

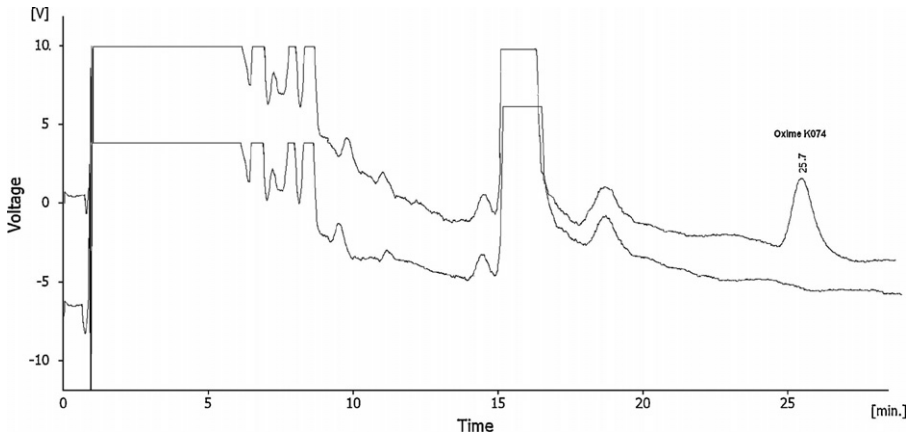

Fig. 3. HPLC chromatogram of oxime K074 in rat brain.

plasma concentrations for precise comparison. Determined results for pralidoxime were 6%, 12%, and 12% for whole brain, cortex and spinal cord, respectively [23], and for obidoxime the measurements ranged between 3 and 5% in all of the brain sections [24].

With the availability of HPLC more exact measurements of oximes may be provided. Sakunda et al. [16] used the microdialysis technique and HPLC for determination of pralidoxime concentration in corpus striatum. The measured extracellular concentration corresponded to 10% of plasma level.

According to the previously published *in vitro* studies on BBB penetration of bisquaternary oximes into the brain, the concentration should be significantly lower. This may be explained by higher molecular weight, higher number of quaternary nitrogens or oxime groups incorporated into the molecule [20,21]. However, several publications did confirm lower concentrations of bisquaternary oximes in the brain tissue: 6% K027 [25] and 3% K203 [26] corresponding to plasma levels. These concentrations have been measured in brain homogenate, through carefully exsanguinated, still contains traces of blood in brain vessels. In the case of much higher plasma concentrations, this may lead to contamination of brain tissue by oximes in blood and subsequently elevate results [2]. Petroianu et al. [27] have shown that partially washed brain tissue overestimated concentration by 15%. In some cases it may be more than 15%; it depends on the process of tissue withdrawing.

Moreover, other experimental data have confirmed that bisquaternary oximes penetrate the BBB in lower concentrations (e.g. 1.3% obidoxime, 0.6% K027, 1.4% K048, compared to plasma concentrations) [2]. Results obtained from the present study were similar. Measured concentrations of trimedoxime and K074 oxime in the brain tissue were 0.58% and 0.85%, respectively, compared to plasma concentrations. The acquired concentrations in the brain tissue were three orders of magnitude lower compared to plasma concentrations. The relatively poor passage of oximes into the brain is most likely related to the hydrophilic properties described by the *in vitro* studies. Unfortunately, these parameters describe only passive permeation through BBB. In reality we have to take into an account carrier systems responsible for the transport of hydrophilic substances into the CNS [28]. Sakurda et al. [16] found possible active transport through the BBB for pralidoxime (monoquaternary AChE reactivator). Interestingly, active transport of bisquaternary oximes into the brain has not been described yet. Other *in vivo* studies have also confirmed poor passage of bisquaternary compounds in to the brain tissue based on monitoring AChE activity. Reactivation was detected only on the peripheral AChE, no activity was detected in the CNS [29,30]. It is clear that reactivation potential is closely linked with the oxime concentration. By *in vitro* and *in vivo* tests significant AChE reactivation was confirmed between 10^{-4} and 10^{-5} M oxime concentrations [31]. However, reactivation potential of bisquaternary oximes in the CNS cannot be completely ruled out since several *in vivo* studies have reported that even low oxime concentrations produced slight AChE reactivation.

Moreover, different brain sections had heterogeneous concentration levels of tested oximes. Highest concentration of both oximes was detected in frontal cortex and lowest in basal ganglia. These outputs closely correspond with *in vivo* reactivation study where highest reactivation was described in frontal cortex and lowest in basal ganglia after application of therapeutic doses of oximes [32,33].

Although reactivation studies are unclear and real oxime concentration in the brain tissue is low slight reactivation effect in the CNS can be expected.

5. Conclusion

In conclusion, AChE reactivators are able to penetrate through the BBB after application of therapeutical doses. The concentrations were low. It seems to that distribution of oximes into the brain obeys some rules, the highest concentration was found in frontal cortex and lowest in basal ganglia. According to the presented *in vivo* data, some slight reactivation effects in CNS should be expected. The reactivation potency of oximes may be higher or lower, depending on the real oxime concentration in a given area.

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