Bioanalytical method development and pharmacokinetics of pyridinium aldoximes using RP-HPLC

Ph.D. thesis booklet

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Introduction

Organophosphates (OPs) as extremely toxic compounds are of high importance in toxicology. According to the WHO statistics more than 1 million of OP intoxications are registered worldwide in every year, caused mainly by inappropriate use of pesticides (e.g. pyriphos). However the nerve gases (e.g. sarin, tabun, VX) are also well known representatives of OPs. The mechanism of action of all OPs is the irreversible inhibition of acetylcholine esterase (AChE) by binding to the hydroxyl group of serin in the active center of the enzyme. The physiological function of AChE is inactivation of the neurotransmitter acetylcholine (Ach) and terminating its effect on acetylcholine receptors in the neuromuscular junctions, vegetative ganglia and parasympathetic postsynaptic receptors as well as in the central nervous system (CNS). Inhibition of AChE results in characteristic symptoms of OP intoxication caused by high excess of Ach both in the peripheral and the CNS.

Nowadays in case of intoxications by organophosphates and organophosphonates beside atropine and diazepam as oxime type antidotes only pralidoxim (PRX) and obidoxim (OBX) are available for clinical use that act by reactivating AChE, however their therapeutic efficacy is far from optimal. To satisfy the permanent need for more effective antidotes synthesis of new antidotes, their *in vitro* and *in vivo* screening is very intensive worldwide.

The research group headed by K. Kuča synthesized a large variety of new AChE reactivator pyridinium and bispyridinium aldoximes (PA and BPA) related to the chemical structure of PRX and OBX. These new aldoximes are common in possessing two pyridinium rings and one or two oxime functional groups. According to the *in vitro* and *in vivo* screening of several bispyridinium bisaldoximes and bispyridinium monoaldoxims the compound K203 [(E)-1-(4-carbamoylpyridinium)-4-(hydroxyimino-methylpyridinium)-but-2-ene dibromide] was found one of the most promising antidote. These new BPAs are very hydrophilic compounds at the physiologic pH because of the two quaterner nitrogens in the pyridinium ring, requiring special bioanalytical method development for pharmacokinetic studies.

Research Objectives

In the frame of an international research project my aims were:

1. To develop such a reverse phase-high performance liquid chromatography (RP-HPLC) bioanalytical method for the most promising K-compounds that is cost-effective, generally applicable and sensitive enough to determine the compound from small biological samples.
2. To determine logP and total polar surface area (TPSA) values of the new K-compounds.
3. Optimization and validation of a RP-HPLC method for K203 using both UV and electrochemical (EC) detections.
4. Determining the pharmacokinetic parameters of K203 in rats and beagle dogs applying the newly developed and validated RP-HPLC method.
5. Studying the blood-brain barrier (BBB) penetration of K203 in rats and beagle dogs using the newly developed validated RP-HPLC method.

Materials and Methods

The PA-s used in our experiment (PRX, OBX, K27, K48, K74, K75, K203, K1000) were synthetically produced by the research group of K. Kuča.

In silico studies

Lipophilicity (logP) and TPSA ($Å^2$) of different K-compouds were calculated using a Pallas program (Pallas 3.8.1.1, CompuDrug International, Inc., Sedona, USA).

Reversed phase high performance liquid chromatography (RP-HPLC)

The method optimization, validation and pharmacokinetic study of K203 was carried out on a JASCO (Tokio, Japan) HPLC system consisting of PU-1580 Pump, DG-2080-54 Degasser, AS-2057 Plus Automatic Injector, UV-1575 UV-Visible and MD-1510 Diode Array detectors. The amperometric/electrochemical detection was done by using INTRO Digital Amperometric Detector (ANTEC Leyden, Zoeterwoude, the Netherlands).

The separation was done using a Agilent Zorbax RX-C18 (250 mm × 4.6 mm, 5-µm) octadecyl silica column coupled with Agilent Zorbax RX-C18 (12.5 mm × 4.6 mm, 5-µm) guard column (Agilent Technologies, supplied by Kromat Kft., Budapest, Hungary). The column oven temperature was 35 °C. The flow rate of the mobile phase was 1 ml/min.

The mobile phase was a mixture of aqueous phosphate-citrate buffer : acetonitrile (10:2 v/v) consisted of 50mM $Na_2HPO_4.2H_2O$, 50 mM citric acid monohydrate; 0.027 mM EDTA disodium salt and varying amounts of 1-octanesulfonic acid sodium salt (OSA). The pH of the mobile phase was adjusted to 3.7 with phosporic acid (85%). The chromatograms were electronically stored and evaluated using Borwin 1.21 and 1.50 chromatograpic softwares (JMBS, Le Fontanil, France).

Validation of the optimized bioanalytical method with both UV and EC detections was carried out according to the internationally accepted (FDA, EMA) guidelines determining selectivity, specificity, accuracy, precision, linearity of calibration curves, limit of detection (LOD), limit of quantitation (LOQ), recovery, robustness, ruggedness and stability.

In vivo pharmacokinetic studies of K203 in biological samples were done using male Wistar rats and beagle dogs. Animals were intramuscularly (i.m.) treated with 3 and 50 µmol/200g K203 freshly dissolved in double-distilled water and were sacrificed between 5 and 240 min

to collect different biological samples. Serum, cerebrospinal fluid (CSF) and also tissue homogenate – brain and different brain areas – were subjected to precipitation using 0.8M perchloric acid (PCA). In order to remove the overwhelming amount of proteins the precipitated homogenates were centrifuged (14 000g, 20 min, 4 °C) and 50 µl of the supernatant were injected into the RP-HPLC system.

Results and Discussion

In silico studies

To develop the HPLC method for the new K-compounds we required the logP and TPSA values of the compounds that were determined *in silico* method using the Pallas software (Table. 1). Based on the chemical structures and the calculated logP and TPSA values it was shown that all the K-compounds studied are very hydrophilic. The two quaterner nitrogen atoms in their pyridinium rings at physiological pH possess two positive charges, so applying appropriate ion-pairing agent is required for separation of peaks in the RP-HPLC system

Table 1. The calculated logP and TPSA values of K compounds (Szegi et al. 2010).

Compound	**TPSA** ($Å^2$)	**logP**
Pralidoxim	36.47	-2.56
Obidoxim	82.17	-2.87
K27	83.44	-2.84
K48	83.44	-2.79
K74	72.94	-2.36
K75	72.94	-2.46
K203	83.44	-3.04
K1000	112.65	-2.3

Optimization of the RP-HPLC method

In the first series of the optimization studies the optimal quantity of different ion-pairing agents was studied and the retention factor (k') was determined. It was found that retention factors for PRX and OBX showed different sensitivity for the concentration of OSA in the mobile phase. The k' value for PRX was less sensitive, while that of OBX was very sensitive for the OSA concentration. Retention factors for K27 (with propylene linker between the pyridinium rigs) and for K48 (with butilene linker between the pyridinium rings) showed low sensitivity for the OSA concentration and that was found for K203 as well. However K203 has a shorter retention time than K74 and K75. These compounds (K74, K75) are symmetric bispyridinium bisaldoximes derivatives of OBX, that may explain the sensitivity of their k' values to OSA concentration.

In the optimization process of the RP-HPLC method the optimal concentration of OSA in the mobile phase could be determined. However when 1 g/l OSA was used in the mobile phase, K203 could not be separated from the background peaks of biological samples (Figure 1.). Increasing the OSA concentration to 2.5 g/l appropriate retention and perfect separation from of K203 was achieved (Figure 2.).

For pharmacokinetic studies sensitivity of the determination should be increased that was performed by EC detection. Electrochemical detection was characterized by 0.2 pmol/inj LOD and 0.68 pmol/inj LOQ values at E_{ox}=+0.8V cell current. This sensitivity allowed determination of K203 from rat brain areas and CSF samples.

When UV detection was used, absorption maximum was at 276 nm and 1.41 pmol/inj LOD and 4.71 pmol/inj LOQ values were achieved.

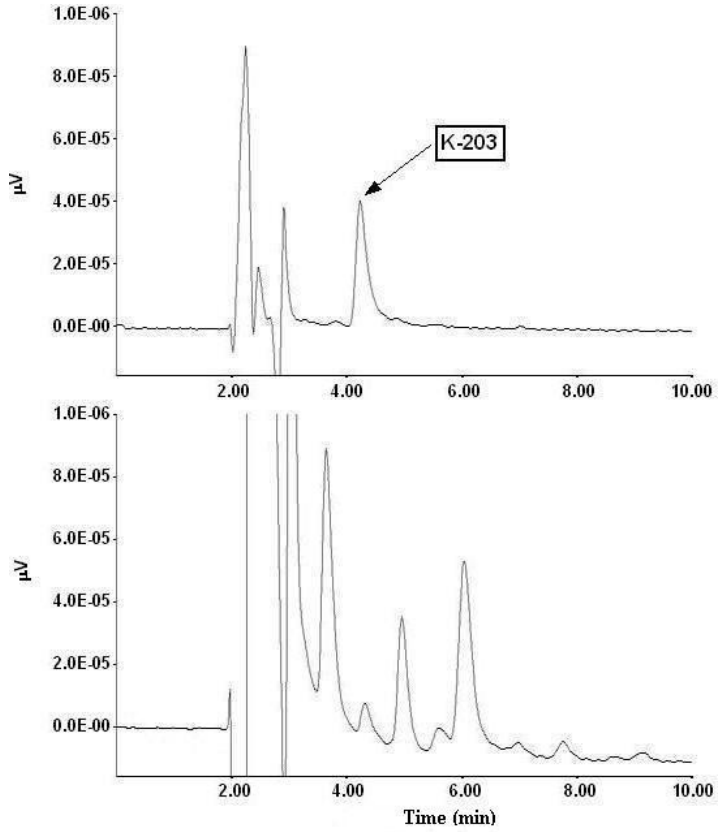

Figure 1. Chromatogram of K203 solution (upper) and that of control rat brain (lower) using 1 g/l OSA and EC detection (Szegi et al. 2010).

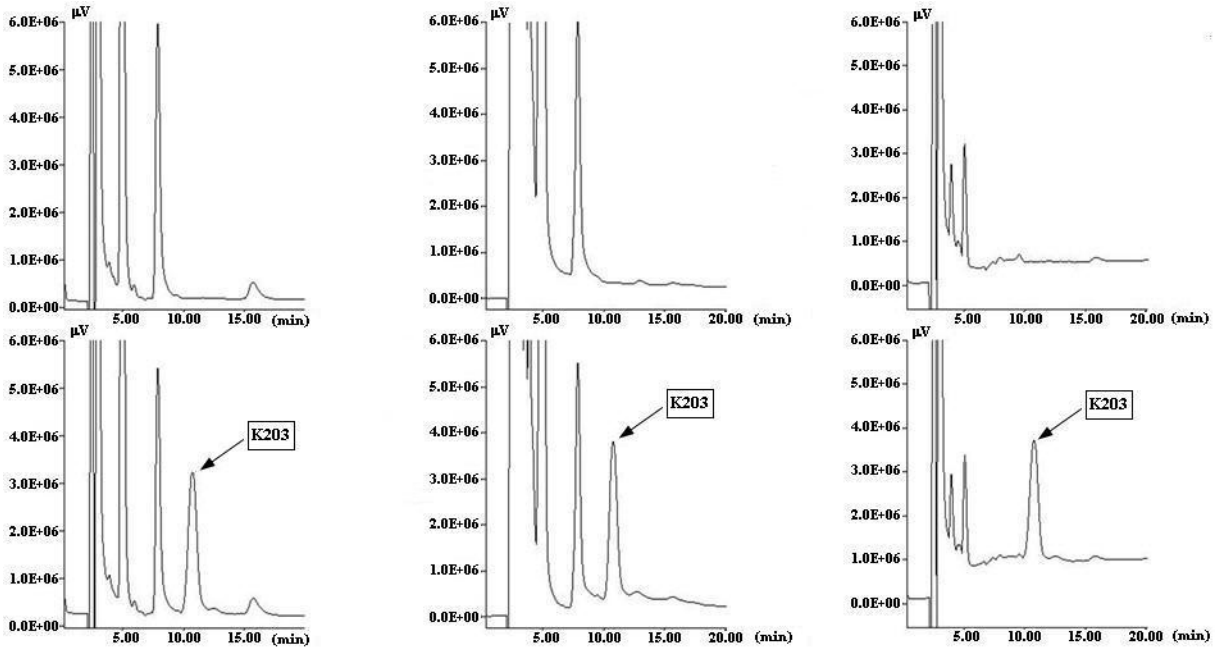

Figure 2. Chromatograms of control rats (upper line) and that of samples spiked with 1µg/ml K203 (lower line) from rat serum, brain and CSF, respectively using 2.5 g/l OSA and EC detection.

Method validation

Using the newly developed and optimized RP-HPLC method K203 could be separated from background peaks of biological matrices and was measured with R_t=9.811 (UV detection) and R_t=10.079 (EC detection) min retention times.

The peak of K203 was found homogenous (homogeneity factor >980) showing that the method is selective for K203 and can be used to determine K203 in different biological matrices. Precision and accuracy values were in the acceptable (93%-113%) ranges given in international guidelines (FDA and EMA). Repeatability values for the method and the chromatographic system suitability values [RSD (%)=0.09] were in the range of 5%.

Calibration curves were linear in the concentration ranges used with r^2 values between 0.9994 – 0.9999. Recovery values were in the range of 86 – 96%. Robustness and ruggedness values showed that the effect of organic modifier (acetonitrile) in the mobile phase is more important that changes in pH values on the retention time of K203.

Stability studies showed significant degradation of K203 in strong acidic solution and at room temperature: in the first 4 hours almost 50% of the compound degradated and at 24 hours only 20 – 22 % of the original concentration could be detected. However at t=4 ºC degradation was significantly lower, at 72 hours 49% of the original concentration was recorded.

Degradation could be significantly decreased at pH 3.7, at 72 hours 91% and 75% of the original concentrations could be recorded at t=4 ºC and t=25 ºC, respectively.

Pharmacokinetics of K203

Tissue concentrations of K203 were determined by the newly developed and validated RP-HPLC method. Rats were injected i.m. with 250 μmol/kg K203. Maximal blood concentration (c_{max}) was recorded at 15 min and half life was found at 45 min. Excretion was via kidneys. The pharmacokinetic values in the eyes were similar to that in the blood (t_{max}: 15 – 20 min, c_{max}: 13 μg/g wet tissue, $t_{1/2}$:60 min).

K203 was determined in both the brain and the CSF samples. It was found that the maximal CNS concentration (t_{max}) is reached at 35 – 45 min and decreases by first order kinetics. Half life ($t_{1/2}$) in the CNS was at 120 min. It is noteworthy that the CSF level of K203 was higher than that of the brain tissue. The brain/blood and CSF/blood concentration ratios calculated showed significant increase in time.

Beagle dogs when injected with a single i.m. dose of 250 μmol/kg K203 showed t_{max} at 20 min and c_{max} of 8.07 ± 2.50 mg/ml ± SD blood level and during the 240 min examination period blood level decreased only wery slowly ($c_{60 - 240\ min}$: 3 – 5 mg/ml). Similar data were obtained in CSF.

When beagle dogs were injected i.m. with 15 μmol/kg K203 t_{max}: 10 – 20 min, and c_{max}: 18.35 ± 2.74 μg/ml ± SD values were obtained and serum level decreased linearly in the 30 – 120 min examination period.

Interestingly, the maximal CSF concentration was reached only at 1 – 2 hours (c_{max}: 1.5 – 1.6 μg/ml).

Conclusions

1. The newly developed and validated RP-HPLC bioanalytical method is applicable for sensitive determination of K203 from different biological matrices (serum, brain, CSF, eyes) with both UV and EC detections.

   The method with minor changes is suitable for bioanalytical determination of other pyridinium aldoximes as well, that are potential new antidotes in organophosphate intoxications.
2. *In silico* studies demonstrated high hydrophilicity of the K-compounds, so their RP-HPLC determination could be done only by appropriate quality and quantity of ion-pairing agent.
3. Having determined the optimal parameters for RP-HPLC of K203 and concentration of ion-pairing agent OSA in the mobile phase the method developed was validated according to the international (FDA, EMA) guidelines.
4. Stability examinations revealed that K203 is degradated in strong acidic solution. Degradation could be avoided:
   a) when tissue samples and samples prepared for HPLC determination were stored at t= -80 ºC
   b) when following protein precipitation using strong acidic solution, the pH of the supernatants were immediately adjusted to 2.0 by diethylamine- phosphoric acid (1:2 v/v) buffer solution
   c) when the temperature in the autosampler chamber was set at t= 4 ºC
5. The newly developed and validated RP-HPLC method was applicable to determine pharmacokinetic data of K203 both in rats and beagle dogs.
6. It was demonstrated that in spite of the very hydrophilic character of K203, the compound can penetrate the BBB in therapeutically effective concentration.

Summary

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K203 is a newly synthesized bispyridinium monoaldoxime type antidote with potential use in organophosphate poisoning, especially in the case of tabun-poisoned subjects.

The aim of our studies was to develop a bioanalytical method for K203 using reverse-phase HPLC (RP-HPLC) technique to measure the tissue levels of K203 in blood serum, brain areas, eyes and CSF for pharmacokinetic studies in rats and beagle dogs. The method could be applicable to determine the blood brain barrier (BBB) penetration of K203 as well.

In the first series of our experiments logP and TPSA values of different K-compounds (K27, K48, K74, K75, K203, K1000) were determined using an *in silico* method. Results confirmed the very hydrophilic character of the compounds. During the method development and optimization of the RP-HPLC method effects of the quantity and quality of ion-pairing agents, methods of sample preparation, effect of temperature, chemical stability of the analyte were determined beside of the optimal wavelength (UV detection) and cell current (electrochemical (EC) detection) for the sensitive determination of K203.

Validation of the optimized bioanalytical method with both UV and EC detections was carried out according to the internationally accepted (FDA, EMA) guidelines determining selectivity, specificity, accuracy, precision, linearity of calibration curves, limit of detection, limit of quantitation, recovery, robustness, ruggedness and stability.

Using the newly developed and validated method pharmacokinetics of low and high doses of K203 were studied in rats and beagle dogs. Time dependence of the concentrations in the plasma, in the CSF, in seven brain areas (hippocampus, hypothalamus, brainstem, medulla oblongata, spinal cord, striatum, frontal cortex) were determined. It was evidenced, that K203 penetrates the BBB in therapeutically effective amount. It was found, that K203 excretion follows a zero order kinetics in case of the high dose both in rats and beagle dogs, however in case of low dose the kinetics follows a first order. It was also shown, that the brain/plasma ratio and the brain/ CSF ratio is changing with the time following the administration.

ÖSSZEFOGLALÁS

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Kísérleteinkben egy, a tabun mérgezés esetén rendkívül hatékony, a terápiás kezelések során antidótumként is alkalmazható, újonnan kifejlesztett PA, a K203 farmakokinetikai paramétereit tanulmányoztuk különböző állatmodellek esetén RP-HPLC módszer segítségével. Erre a célra sikerült egy költséghatékony, optimalizált és validált kromatográfiás eljárást kidolgoznunk.

Vizsgálataink során meghatároztuk a K203 és többi hasonló szerkezetű vegyület logP és TPSA értékeit, amellyel igazoltuk rendkívül hidrofil tulajdonságukat. Az RP-HPLC módszer fejlesztése során az ionpárképző megfelelő koncentrációban történő alkalmazásával sikerült az erősen hidrofil vegyületet fordított fázison elválasztani különböző biológiai minták zavaró háttércsúcsaitól.

Módszerünk validálása során meghatároztuk azokat a paramétereket, amelyek bizonyították alkalmazhatóságát nemcsak a K203, de a többi hasonló szerkezetű PA farmakokinetikai paramétereinek kromatográfiás úton történő meghatározására. A validálás részét képező stabilitás vizsgálatok során megállapítottuk, hogy a K203 savas környezetben erőteljesen bomlik, ezért erre különös figyelmet kell fordítani a kísérletek mintaelőkészítési fázisában valamint a mérés során is. A stabilitásból eredő problémák az általunk leírt ajánlások betartásával kiküszöbölhetőek. Stabilitási vizsgálataimmal hozzájárultam egy megfelelő lejárati idejű gyógyszerformula kidolgozásához.

Az általunk kidolgozott validált módszer egyszerűen és rutinszerűen alkalmazható a K203 szérumból, CSF-ből, valamint agyszövet homogenizátumokból történő mennyiségi meghatározására az állatkísérletek során.

Állatkísérleteinkben patkány és beagle kutya modelleken vizsgáltuk a K203 dózisfüggő kinetikáját a szervezet különböző kompartmentjeiben. Megállapítottuk, hogy a K203 nulladrendű eliminációs kinetikát követve ürül ki a szérumból, miután az i.m. kezelést követően hamar eléri a maximális koncentrációját, ami egyben igazolja az i.m. beadási mód hatékonyságát is. Igazoltuk, hogy a K203 rendkívül hidrofil tulajdonsága ellenére átjut a vér-agy gáton, tehát OP mérgezés esetén a KIR károsodását kivédheti, csökkentheti.

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