# **ORIGINAL ARTICLE**

# Pharmacokinetics of acetylcholinesterase reactivator K203 and consequent evaluation of low molecular weight antioxidants/markers of oxidative stress

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# Summary

Oxime K203 is a new compound designed to be used as an acetylcholinesterase reactivator for the treatment of intoxication following exposure to tabun and certain pesticides. After intramuscular administration of a therapeutic (23 mg/kg) dose, the time-course of plasma concentrations of K203 in rats was determined by HPLC. Maximum concentrations were reached between 40 and 60 min (16.5 $\pm$ 2.1 µg/ml in 40 min and 16.6 $\pm$ 2.0 in 60 min, respectively) with the concentration being relatively constant during this period. There was no significant effect on the plasma concentration of thiobarbituric acid reactive substances (TBARS) during the administration of K203, indicating an absence of oxidative stress. Indeed, administration of K203 led to a significant increase in low molecular weight antioxidants which could tentatively be interpreted as representing a beneficial effect.

Key words: K203; nerve agent treatment; HPLC; plasma concentration; absorption

# INTRODUCTION

Pharmacokinetic studies of acetylcholinesterase (AChE; E.C. 3.1.1.7) reactivators began after the discovery by Wilson and Ginsburg (1955) of their

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benefit in the clinical treatment of poisoning caused by organophosphorus inhibitors (OPI). They also demonstrated that pralidoxime reactivates alkylphosphate-inhibited AChE, and so identified a new class of therapeutic agents (based on pyridinium aldoximes) for the treatment of OPI intoxication (Wilson and Ginsburg 1955). This initial finding was subsequently characterized through ADME (absorption, distribution, metabolism and excretion) studies (Vojvodić and Maksimović 1972, Utley 1987) prior to the adoption of pralidoxime (monopyridinium-mono-aldoxime) as a standard therapy for OPI exposure (Jokanović and Prostran 2009).

The discovery of the efficacy of pralidoxime subsequently stimulated work to synthesise a range of

related compounds such as the bis-pyridiniumbis-aldoximes (e.g. obidoxime, methoxime and trimedoxime) (Kassa et al. 2011). Whilst such compounds exhibit improved efficacy, they do not eliminate associated toxicity (Bartosova et al. 2006). However, further work led to the synthesis of less toxic compounds such as bis-pyridiniummono-aldoxime (HI-6) (Ever et al. 1998) or other untypical structures (Guimarães et al. 2011). According to clinical experience. HI-6 is able to shorten the duration of the respiratory muscle paralysis by reactivation of inhibited AChE. However, the overall efficacy of oximes is still open to debate (Eddleston et al. 2002, Kassa and Karasova 2007, Kassa et al. 2009) and it is conceivable that the mechanism of action of oximes is not mediated solely through the reactivation of inhibited AChE (Pejchal et al. 2008, Pohanka et al. 2009, Soukup et al. 2010).

More recent work includes the synthesis and development of the "K-series" of aldoxime-based AChE reactivators which have a demonstrable ability to reverse AChE inhibition following exposure to certain OPIs (Kalász et al. 2006, Tekes et al. 2006, Petroianu et al. 2007, da Silva et al. 2008, Zdarova Karasova et al. 2009, Kovarik et al. 2009), with improved survival rates (Nurulain et al. 2009). Based on *in vitro* and *in vivo* studies, oxime K203 (bis-pyridinium- mono-aldoxime; Fig. 1) is a promising new reactivator with relatively low acute toxicity which may be of particular benefit in the treatment of tabun poisoning (Kovarik et al. 2009, Kassa et al. 2011).



Fig. 1. Structure of tested oxime.

The purpose of this study was to characterize the pharmacokinetics of oxime K203 following intramuscular (i.m.) administration in the rat. Quantification of plasma K203 concentrations was performed by HPLC. Methods for the HPLC determination of plasma oxime concentrations have been previously described (Brown et al. 1978, Benshop et al. 1981, Guyon et al. 1982, Houze et al. 2005, Zdarova Karasova et al. 2010). We report here a simplified and sensitive method for the

determination of therapeutic concentrations of K203 in plasma which incorporates an improved method for sampling with an oxime preparation based on protein precipitation (Zdarova Karasova et al. 2010). The secondary objective of this study was to assess related changes in antioxidants after the application of oxime K203, by measurement of plasma thiobarbituric acid reactive substances (TBARS), and ferric reducing antioxidant power (FRAP).

# MATERIAL AND METHODS

#### Chemicals

K203 [1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)–but-2-ene dibromide] was synthesized in our laboratory as previously described. The purity of prepared oximes was approximately 98–99% (TLC, mp, NMR) (Musilek et al. 2007). Other chemicals were purchased from commercial sources (Merck, Darmstadt, Germany and Sigma-Aldrich, Steinheim, Germany) and were analytical reagent grade. Solutions were prepared using double distilled, deionized (HPLC grade) water.

#### Instrumentation

Samples were analysed 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 comprising 10 µl loop (Rheodyne, Cotati, USA), an UV1000 detector (Spectra-Physics Analytical, Fremont, USA) controlled by CSW Clarity 2.6.5.517 software (DataApex, Prague, Czech Republic). Separation was achieved using a LiChrospher<sup>®</sup> 60,  $250 \times 4.6$  (5 µm) column distal to a standard guard column (4  $\times$  4 RP-select B; Merck, Damstadt, Germany). The mobile phase was 24% aqueous acetonitrile containing 5 mmol/l octane sulfonic acid and 5 mmol/l tetramethylammonium chloride. The pH was adjusted to 2.3 with phosphoric acid  $(H_3PO_4)$ . The flow rate of the mobile phase was 1 ml/min. Detection was achieved by UV absorption (286 nm) using an UV1000 detector.

#### Animal treatment

The use of animals in this study was approved by the Ethics Committee of the Medical Faculty, Charles University, Czech Republic. Male Wistar rats (Anlab Inc., Prague, Czech Republic) were kept in a climate controlled animal house (temperature  $22\pm2$  °C, humidity  $55\pm6\%$ ) under a 12h:12h light; dark cycle and were allowed access to standard laboratory food and water *ad libitum*.

A total of seven animals were used in this study (average body weight  $320\pm10$  g). After 7 days acclimatization, each animal was anaesthetized by i.p. injection of pentobarbital (administered intraperitoneally; 50 mg/kg body weight) (Novotný et al. 2009). The carotid artery and jugular vein were cannulated to enable serial removal of arterial blood samples (300 µl) or venous replacement of 300 µl saline before administration of the oxime and at regular intervals thereafter (3, 5, 10, 20, 40, 60, 90, 120 and 180 min) (Zdarova Karasova et al. 2011). The oxime was administered via i.m. injection (23 mg/kg; prepared *in situ* using 0.9% saline).

Blood samples were heparinised and the cellular fraction removed by centrifugation (1600 g, 10 min, 4 °C, Universal 320R, Hettich, Germany). The remaining plasma samples were stored at -80 °C prior to analysis.

### Sample preparation for HPLC analysis

Samples of plasma (60  $\mu$ l) were mixed with an equal volume of acetonitrile in order to precipitate proteins. The samples were then spun at 12,000 g at 4 °C for 15 minutes in a centrifuge (M 240R, Hettich, Germany). The resulting supernatant was used immediately for HPLC analysis without any further processing (Zdarova Karasova et al. 2010).

#### Calibration

A calibration curve was established using plasma samples spiked with K203 (1, 6.25, 12.5, 25, 50, 75 and 100  $\mu$ g/ml samples, in triplicates). These were stored at 0 °C for up to 24 hours prior to analysis (which resulted in no detectable decrease in oxime concentration). The retention time of K203 was ~5.3 min.

# *Plasma thiobarbituric acid reactive substances* (*TBARS*)

Samples of plasma (100  $\mu$ l) were mixed with 200  $\mu$ l of 10% (v/v) trichloroacetic acid (TCA) in water. The mixture was incubated for 15 min and spun at 5,000 g for 15 min at 4 °C. The resulting supernatant was then mixed with 200  $\mu$ l of thiobarbituric acid (0.67% w/v) and incubated for 10 min at 100 °C prior to spectrophotometric measurements of absorbance at 532 nm (compared against a blank containing saline).

#### Ferric reducing antioxidant power (FRAP)

A stock solution of reagent was prepared by the addition of 2.5 ml 2,4,6-tris(2pyridyl)-s-triazine (TPTZ; 10 mM, dissolved in 40 mM HCl) to 2.5 ml aqueous FeCl<sub>3</sub> (20 mM), which was then mixed with 25 ml of 0.1 M acetate buffer (pH 3.6). The mixture was incubated at 37 °C for 10 min. Aliquots (200  $\mu$ l)

of the TPTZ reagent were then added to 30 µl of either plasma samples or phosphate buffer prior to mixing in 1 ml distilled water. The resulting solutions were incubated for 10 min at 37 °C and then centrifuged at 10,000 g for 10 min prior to spectrophotometric measurements of absorbance at 593 nm (compared against a phosphate buffer blank).

#### Statistics

Pharmacokinetics profile and calibration curve: The amounts of K203 in each sample were converted to concentration by interpolation of the calibration curve using the data analysis and statistical software Prism4 (Graph Pad Software, San Diego, USA). The pharmacokinetics profile is calculated as mean  $\pm$  standard deviation (n = 7; identical time interval).

FRAP and TBARS statistics: Origin 8 (OriginLab Corporation, Northampton, USA) was used for significance testing by Bonferroni test at the significance level  $2\alpha = 0.05$ .

#### RESULTS

The calibration curve for K203 oxime was linear, in the range of 1–100  $\mu$ g/ml (Fig. 2). Regression analysis (least-squares method) yielded the equation y = 32.03x - 76.58 (R<sup>2</sup> = 0.9931).

Following i.m. dosing, a rapid rise in the plasma concentration of K203 was observed which reached a plateau of  $\sim 16.5 \,\mu$ g/ml between 40 and 60 minutes post administration (Fig. 3). After 60 minutes, the decrease in K203 plasma concentration was confirmed.

There was no significant variation in TBARS plasma concentration during the 3 hour study (Fig. 4), with values ranging from 50 to 90 nmol/l. In contrast, FRAP plasma concentrations steadily increased from  $\sim$ 200 µmol/l after 20 minutes to 490 µmol/l at 180 minutes (Fig. 5), the latter being statistically significant compared with baseline values.

#### DISCUSSION

A range of antidotes are available for the treatment of intoxication caused by exposure to organophosphorus (OP) compounds that act via inhibition of cholinesterase. However, no single antidote has demonstrated universal efficacy against all classes of cholinesterase inhibitors, despite efforts to expand the range of cholinesterase reactivators through the synthesis of novel oximes. Nonetheless, there are a



K203 calibration in plasma

Fig. 2. Calibration plot of plasma samples spiked with K203. The correlation coefficient is 0.9931.



Fig. 3. Plasma level of K203 following intramuscular (i.m.) administration, dose 23.00 mg/kg.

small number of oximes that, with judicial selection, can be used to treat a wide variety of OP intoxication. These include K027 for treating exposure to a range of pesticides (Petroianu et al. 2007) and 2-PAM, HI-6, HLö-7, MMB-4 and K203 as a medical countermeasure for many types of nerve agent.

The oximes HI-6, HLö-7 and MMB-4 are of particular interest due to their relatively wide spectrum of therapeutic activity against nerve agents. Athough they are effective against sarin (GB), cyclosarin (GF), soman (GD) and VX, these oximes are relatively ineffective against tabun (GA) (Berend et al. 2008, Kovarik et al. 2008), possibly due to

reduced access to the active site through steric hindrance resulting from conformational changes induced by the tabun-cholinesterase complex. Consequently, not all reactivators are able to attack the tabun-AChE complex (Ekström et al. 2006). Previous studies have demonstrated that some bis-pyridinium-bis-aldoximes (obidoxime, trimedoxime) are generally more effective for the treatment of tabun poisoning (Zdarova Karasova et al. 2009). However, obidoxime and trimedoxime exhibit increased toxicity compared to K203 (Musilek et al. 2007). Among the new oximes developed, K203 is presently regarded as being highly effective against



Fig. 4. TBAR level in rat plasma. The box indicates standard error of mean, the error bars indicate standard deviation.



Fig. 5. **FRAP level in rat plasma.** The box represents standard error of mean; the error bars indicate standard deviation. Asterisk depicts significance as compared with the start time point (ANOVA with Bonferroni test).

poisoning by tabun and the pesticide paraoxon (Petroianu et al. 2007). The formerly synthesized oximes (e.g. K027, K048, K074 and K075) were developed to increase the effectiveness of treatment in case of tabun poisoning. However, these compounds only attained partial success against tabun. The oxime K203 was prepared by combining the structural features of two relatively effective precursors; K048 and K075 (Musilek et al. 2007), resulting in a bis-pyridinium-mono-aldoxime with a markedly lower inherent toxicity than classical

oximes developed against tabun (Kovarik et al. 2008, Kassa et al. 2011).

Determination of the pharmacokinetics of newly synthesized compounds is an essential step before further (preclinical) investigation. Maximal concentrations after dosage, time to achieve maximal (therapeutic) oxime concentrations and their subsequent elimination kinetics are clearly critical data to acquire in order to ensure candidate compounds are likely to be of clinical relevance. If AChE reactivators are applied i.m., the entire dose is

potentially available for systemic absorption and thus this route of administration offers the highest bioavailability. Oximes are widely distributed in the body and rapidly eliminated by the kidney through secretion by renal tubules (Spöhrer et al. 1994). This study has demonstrated that i.m. administration of K203 results in rapid systemic absorption, with 30% of the maximum plasma concentration  $(C_{max})$ achieved within 3 minutes with a subsequent plateau between 40 and 60 minutes, the calculated  $C_{max}$ corresponding to 49 minute after i.m. application. Similar absorption kinetics were found in a previous study (Kalász et al. 2008) using a higher dose of K203, although elimination at the higher dose was more rapid, being 15% of  $C_{max}$  at 120 min compared to 60% of  $C_{\rm max}$  in this study.

Although the animal treatment, HPLC method and doses (equimolar) were the same, absorption curves of obidoxime and oxime HI-6 were quite different. Oxime HI-6 gave single  $C_{max}$  31 and obidoxime 9 minutes after i.m. injection. The HI-6  $C_{max}$  was similar, only ~16.0 µg/ml and obidoxime  $C_{max}$  was slightly higher ~26.1 µg/ml. Both oximes were eliminated at faster rates (Zdarova Karasova et al. 2010). The other oximes that were studied were trimedoxime and oxime K027. The chemical structures of trimedoxime and K027 are similar. Therefore, it is not surprising that equimolar doses of both oximes resulted in near identical plasma profiles. Trimedoxime 38 minutes (~20.1 µg/ml) after i.m. injection (Zdarova Karasova et al. 2011).

Based on this information, oxime K203 has a better pharmacokinetics profile; the minimal therapeutical concentration was reached 3 min after application and relatively stable plasma concentration was maintained for a long interval of time.

Measurements of low molecular weight antioxidants (FRAP assay) or oxidative stress (TBARS) provide a useful indication of the effect of K203 on the redox state (Pohanka et al. 2011). The apparent lack of effect on TBARS values indicated no significant oxidative stress and the significant increase in plasma antioxidants may be of potential benefit during nerve agent intoxication, although further work should be undertaken to quantify any therapeutic benefits of enhanced antioxidant status during OP-induced cholinergic crises.

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