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ORIGINAL RESEARCH PAPER



A fieldable, high-throughput, cost-efficient high performance liquid chromatography-ultraviolet absorption detection (HPLC-UV) method for the quantitation of bispyridinium quaternary aldoxime cholinesterase reactivators in blood

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ABSTRACT

Mono- and bis-pyridinium quaternary aldoximes (K-oximes) have long been employed as cholinesterase reactivator components of antidotes against lethal cholinesterase-inhibiting organophosphorous chemicals. Their positive charge poses difficulties in their chromatographic analysis, resulting in the publication of different approaches for each K-oxime. A multiplexed method is presented for the rapid quantitation of 10 K-oximes in blood with its utility demonstrated *in vivo*. Liquid chromatography with absorbance detection was employed. Reversed-phase separation was achieved on a highly nonpolar stationary phase. Method validation was based on the respective guideline of the European Medicines Agency. Times to peak concentrations and 120-min areas under the time-concentration curves were determined in rats following intraperitoneal administration. Adequate retention and separation of K-oximes with acceptable peak shapes in short isocratic runs was achieved by adjusting ionic strength, organic content and the concentration of the ion-pairing agent of the mobile phase. Chromatographic properties were governed by optimizing the concentration of dissolved ions. Accurate adjustment of the organic content was indispensable for avoiding peak drifting and splitting. Dose-adjusted exposure to K-347 and K-868 was exceptionally low, while exposure to K-48 was the highest. The method is suitable for screening systemic exposure to various K-oximes and can be extended.

KEYWORDS

pralidoxime, obidoxime, cholinesterase, cholinesterase reactivator, nerve agent

ABBREVIATIONS

EC electrochemical

IS internal standard

LD₅₀ amount of substance which kills 50% of the experimental animals

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LLOQ lower limit of quantification
 MPA mobile phase A
 MPB mobile phase B
 MPC mobile phase C
 OSA 1-*n*-octanesulfonic acid
 QC quality control
 ULOQ upper limit of quantification

INTRODUCTION

Organophosphate pesticides and organophosphonate chemical warfare agents, belonging to the class of nerve agents, are irreversible inhibitors of acetylcholinesterases on account that the amount of acetylcholine increases dramatically at nerve endings, leading to severe clinical symptoms or even death. Poisoning with organophosphates presents a serious and continuous problem worldwide. In addition, recent events have shown that the use of organophosphonate chemical warfare agents in hybrid warfare is still a reality [1, 2].

The most accepted combination treatment protocols of organophosphate and organophosphonate intoxication employ atropine to protect acetylcholine receptors against the overwhelming quantities of acetylcholine, diazepam or midazolam to prevent or decrease the severity of seizures,

and an oxime reactivator [currently pralidoxime, trimedoxime, obidoxime or asoxime (HI-6)] to alleviate acetylcholinesterase blockade [3]. Acetylcholinesterases can be reactivated for a limited time, depending on the nerve agent used, before the toxicant is covalently bound and a subsequent and irreversible structural alteration, called aging, of the product takes place.

To provide the best clinical prognosis for victims, the prophylactic drug or immediate post-exposure administration of an efficient antidote would be imperative. Unfortunately, the effectiveness of current treatment protocols is unpredictable due to the weak effect of atropine on the central nervous system and the poor pharmacokinetic properties of the available acetylcholinesterase reactivators. The optimal reactivator would cross the blood-brain barrier and bind to acetylcholinesterase in a reversible manner, with high rate constants and without causing side effects. The antidote could be a single active agent, a precursor undergoing transformation and giving rise to the effective substance in the central nervous system, or a mixture of molecules.

Quaternary mono- and bis-pyridinium aldoximes, termed as K-oximes, are currently the most potential systemic acetylcholinesterase reactivators (Fig. 1). While sharing close structural similarities, these substances offer a broad range of pharmaco-toxicological profiles [4]. Since the reactivator potencies and the toxicities of K-oximes are closely related to the systemic concentration profiles they

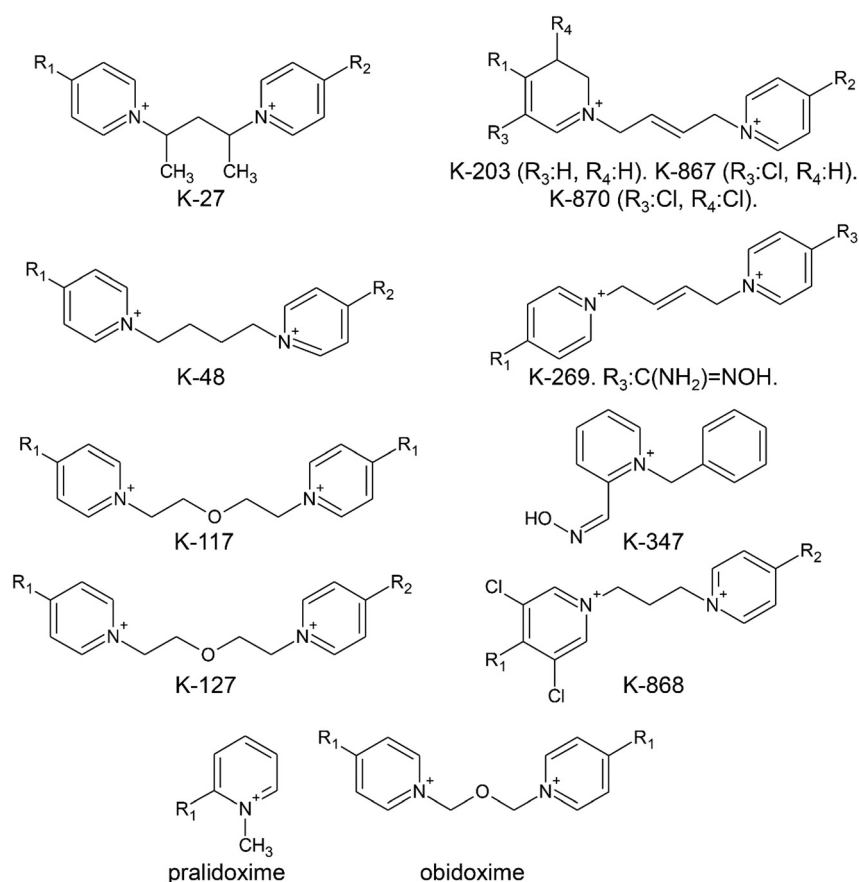


Fig. 1. Structural formulas of the 10 bisaromatic quaternary K-oximes included in this study, and, for comparison, of pralidoxime and obidoxime. Where not indicated otherwise, R₁: CH₂NOH, R₂: CONH₂

attain, and with the emergence of pharmacokinetics-based personalized medicine, monitoring the serum concentrations of K-oximes is a rational perspective. Standardized, cost-efficient analytical approaches deployable in clinical and experimental laboratories should accelerate the related research and facilitate clinical assays.

Quaternary K-oximes consist of up to two aromatic rings, at least one of which is a pyridine containing a quaternary nitrogen, a crosslink between the rings, and at least one aldoxime moiety, in addition to further functional groups. All substances appearing in this work are bispyridinium aldoximes except for K-347, a bisaromatic monopyridinium aldoxime, and have two aldoxime groups (K-117, K-269) or an aldoxime and an amide ligand (K-27, K-48, K-127, K-203, K-867, K-868 and K-870). K-269 contains an α -amino methylamide functional group. The crosslink is a non-branched (K-48, K-347, K-868) or branched alkyl (K-27), or an alkylene (K-203, K-269, K-867, and K-870) residue. The alkyl crosslink of some K-oximes contains an embedded oxygen in ether bond (K-117, K-127). K-867, K-868 and K-870 are mono- or bis-chlorinated derivatives (Fig. 1).

Recently, K-117 and K-127 concentrations have been monitored in blood, cerebrospinal fluid and solid tissue homogenates with a 4-min analysis time [5]. This approach was used for describing the systemic and tissue pharmacokinetics of K-127 *in vivo* [6]. As part of the investigation of the pharmacokinetics of several novel K-oximes, our aim is to present a high-throughput, cost-efficient, multiplexed approach relying on high performance liquid chromatography (HPLC) and ultraviolet (UV) detection for the quantitation of an extendable set of analytes in serum, and to demonstrate its utility in pharmacokinetic experiments.

EXPERIMENTAL

Chemicals and solutions

The dibromide salts of K-27, K-48, K-117, K-127, K-203, K-269, K-867, K-868, and K-870, and K-347 monobromide were contributed by the Department of Chemistry, Faculty of Sciences, University of Hradec Králové (Hradec Králové, Czech Republic). Acetonitrile LC-MS grade, citric acid hydrate a.r., methanol LC-MS grade, sodium acetate a.r., and water LC-MS grade were purchased from Molar Chemicals Kft (Halásztelek, Hungary). Perchloric acid 70%, puriss., p.a., ACS grade, was bought from Sigma-Aldrich Kft (Budapest, Hungary). Sodium octanesulfonate 99% was obtained from Reanal Labor Kft (Budapest, Hungary).

2.0 mg/mL stock solutions of the K-oximes were in methanol–water (1:1, v/v) and stored at -75°C . 0.3 mol/L perchloric acid was prepared in water. Internal standard (IS) solutions contained 100 $\mu\text{g/mL}$ of K-347 or K-870 in water.

In vivo experiments

3 μmol (K-269, K-347) or 30 μmol (K-867, K-868 and K-870) were administered as a 0.2 mL intramuscular bolus

injection to Wistar rats (male, 185–195 g, Toxi-Coop, Budapest, Hungary) in an aqueous solution. Each drug was evaluated using 11 animals, 10 of which were sacrificed at 5, 15, 30, 60, or 120 min ($n = 2$ each). The 11th animal was left untreated. Experiments were conducted at the Department of Pharmacodynamics, Semmelweis University, in line with the Guide for the Care and Use of Laboratory Animals [7], and granted the permission of the Governmental Office of Pest County, Hungary (PE/EA/385-5/2018).

Biological specimens

The serum of untreated rats, allocated for disposal, was used for method development and validation. Stock solutions or the aqueous working solutions of the analytes (≤ 25 μL) were spiked to 1.0 mL aliquots of the serum specimens to obtain quality control samples. Calibrator samples were prepared in the same manner using pooled rat serum. Blood samples collected in the *in vivo* experiments were centrifuged immediately at 4°C ($21,952 \times g$, 20 min), and at least 125 μL serum was separated and frozen to -75°C until analysis.

Sample preparation

100 μL serum was diluted with 890 μL of 0.3 mol/L perchloric acid after adding 10 μL internal standard solution. The mixture was centrifuged at ambient temperature ($19,000 \times g$, 5 min) and the supernatant was submitted for analysis.

Analysis

Analysis was accomplished on a JAS.CO 4000 high performance liquid chromatography system (PU-4180 pump, AS-4050 sampler with a TC-4000 sample tray cooled to 8°C , and an MD-4010 photodiode array detector). The analytical column was thermostatted at 40°C . Instrument control and data acquisition was performed using the ChromNav 2.0 software (ABL&E-Jasco Hungary Kft, Budapest, Hungary).

The stationary phase was Phenomenex Kinetex EVO C18 (100×3 mm, particle size: 5 μm , Gen-Lab Kft, Budapest, Hungary). The mobile phase contained an aqueous buffer (80%), acetonitrile (10%) and methanol (10%). The aqueous buffers tested during method development (MPA, MPB, and MPC) contained 4.72 g/L citric acid monohydrate, 3.7 g/L sodium octanesulfonate, as well as 9.03 g/L, 18.06 g/L, or 36.12 g/L of sodium, acetate, trihydrate, respectively. The robustness of the chromatographic properties of the analytes was tested using MPB–acetonitrile–methanol 82:9:9 and MPB–acetonitrile–methanol 78:11:11. Separation was achieved under isocratic conditions. The mobile phases selected in the validation phase were MPB–acetonitrile–methanol 80:10:10 (K-27, K-48, K-117, K-127 and K-269) or MPC–acetonitrile–methanol 80:10:10 (K-203, K-347, K-867, K-868 and K-870). The mobile phase flow rate was 0.8 mL/min. The injection volume was 20 μL . The detector was operated in the multiple wavelength mode set to 270 nm (K-868), 280 nm (K-27, K-48, K-117, K-127, K-203, and K-269), and 300 nm (K-347,



K-867, and K-870). The run time of the final method was 10 min.

Data evaluation

Retention factors of the analytes were calculated using the equation $(t_r - t_0)/t_0$, where t_r is the retention time of the analyte and t_0 is the hold-up time of the analytical system (0.5 min). Peak asymmetry was calculated by the ChromNav 2.0 software. K-347 was employed as the IS for K-27, K-117, K-127, K-867, K-868, and K-870. K-48 and K-347 were quantitated using K-870 as the IS. Quantitation was performed by calculating the peak area ratios of the analytes and the internal standards, in calibrators and in the samples. The target calibration concentration range was 0.5–100 µg/mL with respect to K-oxime bromide salts. $1/x^2$ -weighted linear calibration models were fitted using the `lm()` function of the 'stats' package of R [8]. Statistical evaluation was performed using Microsoft Excel 2013. *In vivo* areas under the curve were calculated by applying the trapezoid method to data pairs of time and mean concentrations obtained in the two parallel experiments [9].

Method validation

Method validation was based on the respective guideline of the European Medicines Agency [10]. Selectivity was verified by assaying seven blank serum specimens following their preparation as described above. Analyte carry-over was assessed by running the highest-level calibrator of each analyte three times with blank solvent runs in-between. Robustness was evaluated by changing the organic content of the mobile phase by $\pm 2\%$. Within-run accuracy and precision were evaluated using six independent serum matrices and four quality control (QC) levels using the following target concentrations: QC1, 125% and QC2, 250% of the lower limit of quantitation (LLOQ), as well as QC3, 45% and QC4, 90% of the upper limit of quantitation (ULOQ). The LLOQ and the ULOQ were defined as the lowest (0.5 µg/mL) and highest (100 µg/mL) calibrator levels, respectively. Between-run accuracy and precision were assessed by running QC1–QC4 prepared in a single serum using independent calibrator series on three consecutive days. Autosampler stability was addressed by leaving a calibrator sample (target bromide salt concentration: 5 µg/mL) in the sample tray at 8 °C and injecting it repeatedly every 60 min for 24 h. Freeze-thaw stability was investigated in three cycles on a QC2 and a QC4 sample. The evaluation of autosampler stability was based on analyte peak areas, while freeze-thaw stability was assessed by determining analyte concentrations in the samples. All calibration, quality control and experimental samples were diluted ten-fold, therefore, dilution integrity was not evaluated in separate experiments.

RESULTS

The employed stationary phase and the mobile phases gave rise to an acceptable retention of the analytes and their

separation from the respective internal standards as well as from interfering peaks (Fig. 2). The developed approach demonstrated acceptable selectivity for the analytes. No carry-over was observed. Although a single detection wavelength optimal for all analytes could not be defined, quantitation could be accomplished at 270, 280, or 300 nm. The concentration of sodium acetate in the aqueous component of the mobile phase had a fundamental impact on the retention, the signal intensities and the peak asymmetry of the analytes (Table 1). Representative chromatograms are displayed in Fig. 2.

Decreasing the organic content to 8% resulted in an increase of the retention factors by 27–108%. Increasing the organic content to 12% caused the retention factors to drop by 29–49%. This impact of the organic component was least pronounced in the case of K-203 and K-868 (Fig. 3).

The target retention factor range of the analyte peaks was 5–19 (see Discussion section). Due to heavy retention (K-867, K-868, and K-870), peak splitting (K-27, K-48, K-127, and K-203) or very low signal intensities (K-117, K-269, and K-347), MPA could not be employed for the reliable quantitation of the analytes. Based on their retention factors and improved peak symmetries, however, the serum concentrations of K-27, K-48, K-117, K-127, and K-269 could be determined optimally using MPB. As an exception, the splitting of K-203 peaks could only be prevented by employing MPC. While their retention was acceptable when MPB was used, further improvement was achieved by using MPC for both regarding the retention and the signal intensities of K-867 and K-870. The retention of K-868 was acceptable only when MPC was used. The chromatographic properties of K-347 were acceptable regardless of the mobile phase used, with some improvement in signal intensities observed in association with increasing the buffer concentration. As a result, MPC was used for the quantitation of K-203, K-347, K-867, K-868, and K-870.

The outcomes of the within- and between-run accuracy and precision experiments are displayed in Table 2. The results of the autosampler tray and freeze-thaw stability tests are presented in Tables 3 and 4, respectively.

The results of the *in vivo* experiments performed using K-269, K-347, K-867, K-868, and K-870 are shown in Table 5. Similar studies with K-27, K-48, K-117, K-127 and K-203 had been conducted earlier by our group, therefore, their results are also displayed [6, 11–13]. In the present work, the highest serum concentrations of K-868 and K-870 were detected at 15 min. The peak levels of K-269 and K-867 were observed by 30 min. The maximum concentration of the mono-quaternary aldoxime K-347, however, was already attained in the first sample drawn at 5 min.

Eventually, two distinct groups of the investigated bis-quaternary aldoximes could be differentiated by their 120-min dose-adjusted systemic exposure: a 'high exposure' group comprising K-27, K-48, K-117, K-127, K-269, and K-867 [414–939 µg min/(mg mL)], and a 'low exposure' group



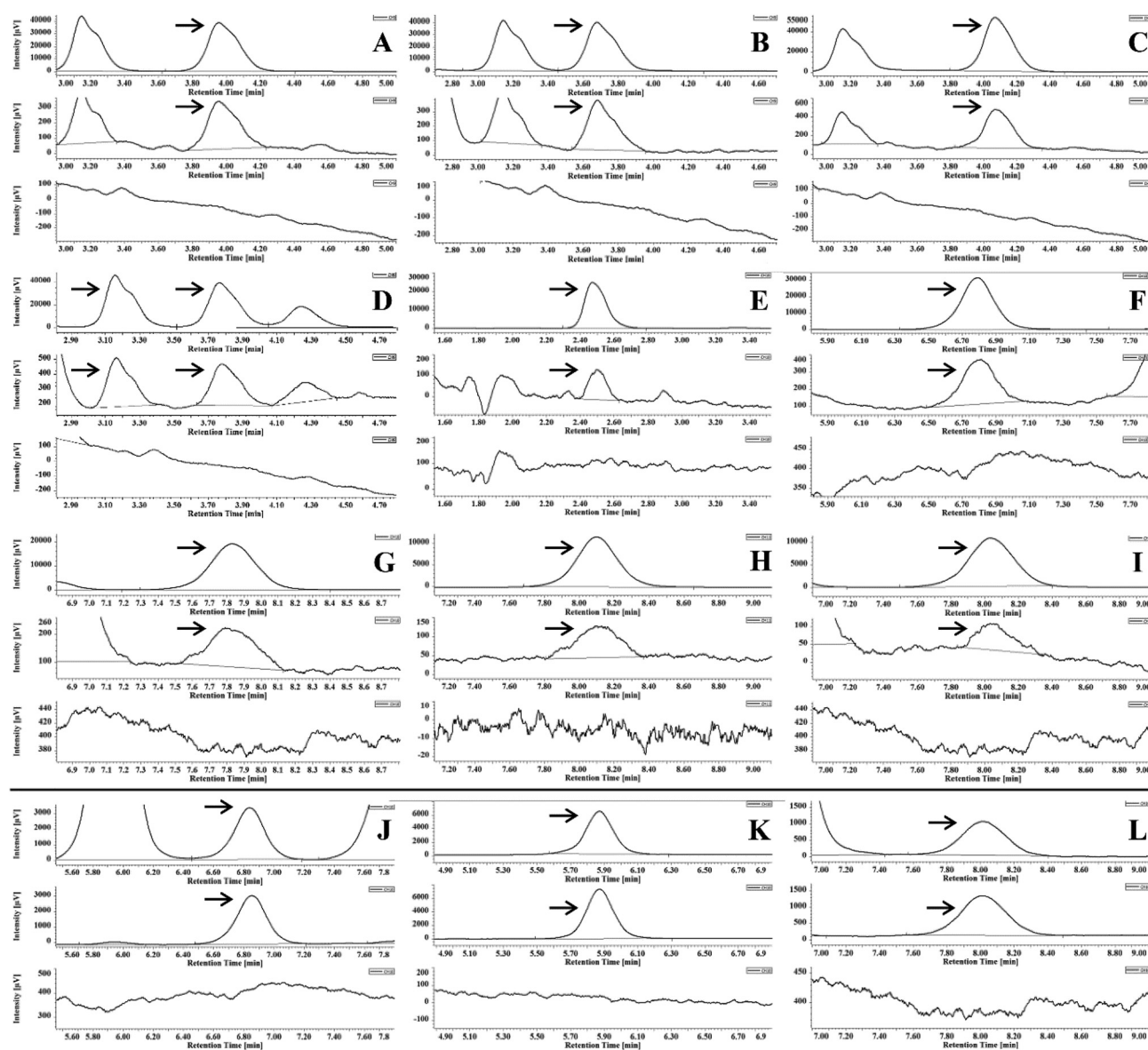


Fig. 2. Representative chromatograms of the investigated bisaromatic quaternary K-oximes. (A) MP-B, 280 nm. K-27: 4.0 min. (B) MP-B, 280 nm. K-48: 3.7 min. (C) MP-B, 280 nm. K-117: 4.1 min. (D) MP-B, 280 nm. K-127: 3.2 min, K-269: 3.8 min. (E) MP-C, 300 nm. K-203: 2.5 min. (F) MP-C, 300 nm. K-347: 5.9 min. (G) MP-C, 300 nm. K-867: 5.2 min. (H) MP-C, 270 nm. K-868: 9.5 min. (I) MP-C, 300 nm. K-870: 5.3 min. (J) MP-B, 300 nm. K-347 (internal standard): 6.8 min. (K) MP-C, 300 nm. K-347 (internal standard): 5.9 min. (L) MP-B, 300 nm. K-870 (internal standard): 8.0 min. Top: Level 4 quality control sample. Middle: Level 1 quality control sample (lower limit of quantitation). Bottom: blank serum. MP-B, mobile phase 'B'. MP-C, mobile phase 'C'

including K-203 and K-870 [262–271 $\mu\text{g min}/(\text{mg mL})$]. Exposure to K-347 and K-868 was considerably lower [61.2 and 95.8 $\mu\text{g min}/(\text{mg mL})$, respectively] than those recorded for all of the other substances.

DISCUSSION

Earlier methods published on the evaluation of the pharmacological and toxicological properties of quaternary K-oximes have all used HPLC with EC or UV detection for the analysis. In the only available methodological work on a wide range of K-oximes, the

chromatographic properties of pralidoxime, obidoxime, K-27, K-48, K-74, K-75, K-203, and K-1000 were investigated, but even here only K-203 was tested in biological specimens [14]. All of the approaches used isocratic reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet absorbance (UV) or electrochemical (EC) detection, and octyl- or octadecylsilica stationary phases. Octylsilica phases were employed with mobile phases containing 80% pH = 2.6 aqueous solution of 1-*n*-octanesulfonic acid (OSA) and 20% acetonitrile, without any buffer. The preparation of serum samples consisted of protein precipitation [15–17]. A mobile phase containing pH = 2.6 aqueous solution of OSA and 8%

Table 1. The impact of the mobile phase buffer strength on analyte retention and peak characteristics. The mobile phase was an aqueous buffer–organic solvent mixture (80:20, v/v). The buffer concentration in the mobile phase was 0.062 mol/L (MPA), 0.124 mol/L (MPB) or 0.186 mol/L (MPC). Means of three measurements are shown

Identifier	MPA			MPB			MPC		
	Retention factor	Relative peak intensity (%)	Peak asymmetry	Retention factor	Relative peak intensity (%)	Peak asymmetry	Retention factor	Relative peak intensity (%)	Peak asymmetry
K-27	11.58	100	split peak	6.80	96.7	1.35	5.2	90.4	1.07
K-48	10.7	72.0	split peak	6.40	88.2	1.34	4.8	100	1.04
K-117	13.2	27.9	0.925	7.14	48.5	1.17	4.58	100	1.24
K-127	9.56	34.5	split peak	5.34	65.2	1.27	3.52	100	1.30
K-203	12.5	41.6	split peak	6.36	70.2	split peak	4.48	100	1.34
K-269	12.0	49.9	1.04	6.52	68.9	1.23	4.24	100	1.29
K-347	16.1	58.7	0.99	12.5	75.7	1.03	9.82	100	1.05
K-867	25.6	32.0	0.98	14.5	53.8	1.01	9.34	100	1.07
K-868	46.8	34.8	1.14	23.2	69.6	1.04	18.0	100	0.96
K-870	26.6	30.8	1.06	14.9	52.8	1.00	9.60	100	1.06



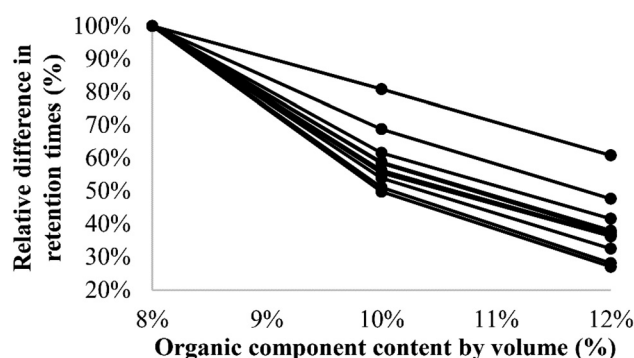


Fig. 3. Impact of the organic component on the retention times of the investigated K-oximes

methanol as the organic component was employed with UV detection [11, 12, 18, 19]. Octylsilica stationary phases were employed along with aqueous buffers containing a phosphate–citrate buffer, disodium–edetate and OSA. The organic component was 7–17% acetonitrile. In these experiments, serum was diluted ten- to twenty-fold by adding diluted perchloric acid, a process which included the precipitation of serum proteins [14, 20–23].

In order to attain acceptable selectivity and to allow short runs, we selected a retention factor of 5–19 as a target range for the analytes. Even minor changes in chemical structure gave rise to substantial differences in the chromatographic properties. To reduce the number of factors influencing retention, an end capped, ethane cross-linked, core-shell

Table 2. Results of the within-run and between-run accuracy and precision study

Substance	Concentration	Within-run		Between-run	
	($\mu\text{g/mL}$)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
K-27	0.475	102	18.0	99.7	10.2
	0.809	94.3	8.08	112	14.8
	28.2	99.0	6.24	100	3.7
	60.3	97.4	3.73	99.3	3.8
K-48	0.483	99.5	10.0	87.0	13.5
	0.822	92.9	9.75	98.2	7.10
	28.7	103	11.0	85.2	2.61
	61.4	104	6.48	101	4.51
K-117	0.492	94.5	19.2	101	13.0
	0.837	107	8.16	105	695
	29.2	102	2.21	100	3.18
	62.5	99.3	2.79	102	2.50
K-127	0.492	87.5	14.1	98.4	10.2
	0.837	92.1	5.95	102	7.49
	29.2	108	2.12	103	5.67
	62.5	109	1.46	102	5.48
K-203	0.391	87.4	6.63	91.9	18.8
	0.781	93.3	8.22	89.9	8.0
	29.3	111	7.67	98.8	10.3
	58.6	110	10.9	95.4	10.5
K-269	0.490	95.1	16.6	98.0	10.0
	0.834	96.4	7.91	103	8.58
	29.1	104	1.74	101	5.02
	62.3	106	1.49	100	4.46
K-347	0.538	93.6	10.0	99.0	10.4
	0.917	99.4	5.41	106	14.4
	32.0	103	11.3	88.1	3.92
	68.4	107	3.72	104	5.21
K-867	0.500	106	19.5	103	18.0
	0.851	93.8	7.62	112	14.3
	29.7	103	6.59	105	3.72
	63.5	103	4.26	104	3.16
K-868	0.414	110	14.5	117	20.0
	0.828	97.9	9.57	95.6	10.9
	31.0	109	8.71	100	1.6
	62.1	108	11.8	95.5	4.0
K-870	0.516	107	12.0	99.4	9.81
	0.878	115	7.80	108	11.8
	30.7	106	3.16	103	6.52
	65.5	102	2.83	103	5.87

Table 3. Results of the autosampler stability study. A calibrator sample was kept at 8 °C and injected repeatedly every 60 min

Time (h)	K-27	K-48	K-117	K-127	K-203	K-269	K-347	K-867	K-868	K-870
0	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
1	100.0%	99.2%	100.0%	99.4%	99.2%	99.4%	99.3%	99.9%	100.3%	100.7%
2	99.3%	100.4%	99.4%	99.0%	98.7%	98.6%	100.5%	100.1%	102.7%	100.3%
3	98.9%	102.1%	99.1%	98.6%	98.0%	97.9%	101.0%	99.8%	100.1%	100.0%
4	98.5%	102.6%	98.2%	97.8%	96.4%	97.0%	100.6%	99.4%	102.5%	100.6%
5	97.9%	102.3%	97.6%	97.5%	95.6%	96.5%	100.8%	99.7%	99.5%	99.6%
6	97.6%	101.6%	97.2%	96.9%	94.2%	95.9%	98.8%	99.4%	97.3%	99.9%
7	96.8%	100.2%	96.5%	95.9%	93.1%	94.5%	98.5%	99.9%	97.3%	99.4%
8	97.1%	100.2%	96.2%	95.0%	92.4%	93.5%	98.1%	98.7%	98.4%	99.4%
9	95.7%	99.7%	95.3%	94.2%	91.2%	92.5%	97.2%	99.0%	97.2%	99.4%
10	95.3%	97.6%	94.7%	93.3%	89.3%	91.5%	95.3%	98.5%	96.5%	99.5%
11	93.3%	97.4%	94.3%	92.5%	87.8%	90.5%	95.1%	98.3%	95.6%	99.4%
12	94.3%	97.4%	93.9%	91.5%	87.1%	89.4%	95.9%	98.3%	93.9%	99.0%
13	93.5%	96.1%	93.6%	90.3%	86.1%	87.9%	95.0%	97.7%	94.1%	98.8%
14	93.6%	95.4%	92.7%	88.8%	84.4%	86.5%	93.5%	98.1%	90.8%	98.7%
15	92.7%	95.2%	92.1%	88.1%	83.4%	85.6%	92.2%	97.5%	92.1%	98.7%
16	92.2%	94.7%	91.6%	87.5%	82.5%	84.9%	91.1%	97.3%	90.9%	99.2%
17	90.0%	93.0%	91.0%	87.2%	82.3%	84.5%	91.9%	97.9%	91.9%	99.0%
18	91.0%	92.4%	90.4%	85.6%	82.0%	82.7%	93.2%	97.0%	91.0%	99.2%
19	90.5%	93.1%	89.8%	84.2%	80.5%	81.1%	91.0%	96.6%	90.4%	98.6%
20	90.4%	92.3%	89.4%	83.1%	80.7%	80.0%	90.3%	96.7%	89.7%	98.0%
21	89.8%	90.4%	88.7%	82.6%	79.7%	79.4%	90.5%	96.3%	93.0%	97.7%
22	89.3%	90.4%	88.2%	82.1%	79.0%	78.8%	91.2%	95.9%	89.8%	97.7%
23	88.7%	89.9%	87.6%	81.7%	78.6%	78.3%	90.2%	96.5%	90.0%	98.5%
24	88.3%	90.0%	87.3%	79.6%	78.3%	76.1%	91.3%	96.0%	90.7%	98.0%

octadecylsilica stationary phase packed in a short, small internal diameter analytical column was used. Therefore, target retention factors could be attained by using a weak mobile phase, leaving little room for intervention by adjusting its

organic content. Preliminary experiments showed that, in order to obtain adequate retention and peak shapes with acceptable reproducibility for all analytes, the organic component had to contain both methanol and acetonitrile.

While bisaromatic quaternary K-oximes are retained well by reversed-phase systems following the formation of ion pairs, their peaks are prone to shouldering or even splitting if the overall ion concentration is insufficient. Using mobile phases with various buffer concentrations not only affected

Table 4. Results of the freeze-thaw stability study (−18 °C, 3 cycles, $n = 3/\text{cycle}$)

Substance	Nominal concentration (µg/mL)	Mean measured/nominal concentration (%)
K-27	0.809	108
	60.3	96.2
K-48	0.822	103
	61.4	98.8
K-117	0.837	108
	62.5	95.5
K-127	0.837	99.5
	62.5	99.2
K-203	0.391	83.2
	58.6	90.4
K-269	0.834	109
	62.3	98.2
K-347	0.917	110
	68.4	99.5
K-867	0.851	99.3
	63.5	102
K-868	0.414	115
	62.1	97.6
K-870	0.878	108
	65.5	98.7

Table 5. Pharmacokinetic properties of the K-oximes included in the present analytical method. K-269, K-347, K-867, K-868 and K-870 were evaluated in the present work. $T_{\max}$, time to reach peak concentration. AUC_{0-120} , area under the curve in the interval of 0–120 min following administration of the test substance.

Substance	Dose (mg)	$T_{\max}$ (min)	AUC_{0-120} (µg min/mL)	Dose-adjusted AUC_{0-120} [µg min/(mg mL)]
K-27 [12]	22.3	15	14,198	637
K-48 [11]	23.0	15	21,598	939
K-117 [6]	1.43	15	775	542
K-127 [6]	1.43	15	931	651
K-203 [13]	22.9	15	6,005	262
K-269	1.42	30	848	597
K-347	0.879	5	57.6	65.5
K-867	14.3	30	5,918	414
K-868	15.5	15	1,485	95.8
K-870	15.8	15	4,279	271



peak shapes but also allowed proper intervention into the retention characteristics of the analytes. Using MPA, the mobile phase with the smallest buffer concentration, resulted in an extremely heavy retention of the chlorinated quaternary oximes K-867, K-868, and K-870, as well as the splitting of the peaks of the non-halogenated oximes containing an oxime and an amide moiety (K-27, K-48, K-127 and, K-203). K-117, a non-halogenated quaternary oxime containing two oxime ligands was eluted with an acceptable peak shape (asymmetry factor of 0.92). The intensities of all peaks, except for K-27, were also considerably smaller than those obtained by employing MPB or MPC. Use of MPB improved the peak shapes of K-27, K-48, and K-203, increased the signal intensities without increasing the background noise, and brought the retention factors of the analytes, except for K-868, into the target range. The peak of K-203 remained, however, splitted, and the symmetry of the K-269 peak deteriorated. Using MPC resulted in the retention factors of the non-halogenated bipyridinium aldioximes K-48, K-117, K-127, K-203 and K-269 to drop below 5, while that of K-868 was now in the target range. The peak of K-203 could be detected without splitting and shapes of the other peaks were acceptable, except for those of K-117 and K-269 which showed deterioration. Further improvement in signal intensities was also obtained.

We conclude that the presence of heteroatoms in the crosslink or in the aromatic rings, as well as the replacement of the oxime ligand with an amide or an α -amino amide moiety lead to decreased retention of the ion pairs of bisaromatic quaternary K-oximes in reversed phase systems. Double bonds and branches in the crosslinks have a minor impact. Peak shouldering and splitting were characteristics of the non-halogenated bipyridinium oxime amides at low, as well as of the bipyridinium bis-oxime K-117 and the oxime- α -amino amide K-269 at high buffer concentrations. Using MPB yielded acceptable retention factors and peak shapes for the non-halogenated analytes, while MPC proved to be optimal for the chromatography of K-203, a non-halogenated oxime-amide with a double bond in the crosslink, as well as for the halogenated substances. The investigated variables exerted the smallest impact on the bis-aromatic, monopyridinium aldoxime K-347. It must be noted that injection volumes exceeding 20 μ L also led to poor and inconsistent peak shapes.

The slightest changes in the organic content of the mobile phase have a substantial impact on the reproducibility of the assay (Fig. 3). Mobile phases with a low organic content may therefore lead to inconsistencies in the analysis of quaternary K-oximes due to the evaporation of the organic component. Over time, this may lead to lower signal intensities, compromising the evaluation of *in vivo* pharmacotoxicokinetic experiments performed on these substances. Consequently, employing a standardized technical procedure for preparing the mobile phases is recommended.

With the only variable component being the mobile phase (MPB or MPC was employed as detailed above), the quantitation of all analytes with acceptable accuracy and precision in the range of 0.390–65.5 μ g/mL (corresponding

to 0.5–10 μ g/mL of the bromide salts of the analytes) was accomplished. In solutions, 0.4 ng was established universally as the limit of detection.

The quantitation of quaternary K-oximes has been performed using internal standards only recently [5, 6]. In the current study, K-347 proved to be suitable as IS for the entire range for the analytes, regardless of which mobile phase was used, except for K-48. K-48 and K-347 were eventually assayed using K-870 as the IS.

The results of the autosampler stability test and the freeze-thaw study indicate that the stability of K-203 is lower than that of all other analytes (Tables 3 and 4). This finding confirms the results of Szegi et al. [14] and warrants research of its stabilization in biological specimens and in acidic extraction media. The analysis of K-127 and K-269 also needs careful timing as their relative peak areas fall below 85% by 19 and 16 h, respectively. All other analytes exerted acceptable stability over 24 h.

Two basic approaches are applied widely in preclinical pharmacokinetic studies: serial blood sampling of animals, and sacrificing them at predetermined time points to obtain information about the distribution of the compound studied. The minimal number of the animals to be sacrificed for gaining reliable data is provided by well-known statistical methods taking in consideration, among others, method validation results. Both approaches were tested in our previous studies, however, no appreciable difference was found. Moreover, certain issues such as the characteristics of the penetration of a compound through the blood-brain barrier, which are essential for the determination of a potential carrier molecule or therapeutical value of the compound studied, require the approach of sacrificing the animals in different times following treatment. Therefore, this approach has been selected for out investigations [24].

Currently, manufactured oximes (pralidoxime and obidoxime) reach peak levels extremely fast (within 5 min) and are also eliminated rapidly, with a half-life of about 40 min [25]. The concentration profile of the monopyridinium aldoxime K-347 was in line with this finding, while the bipyridinium aldioximes attained peak levels only at 15–30 min. This is important in terms of the onset of reactivating effects (n.b. all tested substances were detected at concentrations higher than the lower limit of quantitation in the 5-min serum samples) and the toxicity of the K-oximes, which correlates with their intrinsic *in vitro* acetylcholinesterase inhibitory activity. K-269 has been found to be more toxic than obidoxime, and exposure to this substance is considerable and long-lasting [26]. In contrast, the LD₅₀ of K-347 in rats was higher than that of obidoxime and comparable to that of HI-6 [27]. The pharmacokinetic characteristics of K-347, specifically, the attainment of peak levels at 5 min and the very low dose-adjusted area under the concentration–time curve, also indicate that higher safety can be expected when administering this oxime in comparison with the rest of the tested substances. In contrast, exposure to K-48, an aldoxime with an LD₅₀ similar to that of obidoxime, was much larger than



that of the rest of the tested compounds, warranting caution when considering its use.

Very recently, ortho-dichlorinated bispyridinium aldoximes have been demonstrated to have a highly superior reactivating efficacy on butyrylcholinesterase in comparison to that of K-27. K-868 has been especially promising [28]. An additional favorable property of K-868 is the relatively low exposure accompanied by a relatively late attainment of its maximum serum concentration. These properties may allow the targeted guidance of its administration. The time required for K-867 to reach peak levels was one of the highest observed (30 min), with an intermediate dose-adjusted area under the time–concentration curve (Table 5).

CONCLUSIONS

A wide range of bisaromatic quaternary mono- and bispyridinium aldoxime cholinesterase reactivators can be quantitated successfully in blood using the presented isocratic reversed-phase HPLC-UV system with the only adjustable setting being the buffer concentration in the mobile phase. The investigated K-oximes exert acceptable stability in serum and in diluted perchloric acid, the medium most often employed for serum protein precipitation and for the extraction of these analytes from solid tissues, except for K-127, K-269, and K-203.

A standardized approach to the bioanalysis of these substances facilitates multi-center pharmaco- and toxicokinetic studies, and contributes to the establishment of clinical protocols for the safe and efficient treatment of the victims of intoxication with organophosphates or victims of nerve agent attacks. The presented method is prone to standardization and can be implemented in experimental and clinical laboratories having access to basic HPLC equipment without requiring special expertise or training.

Conflicts of interest statement: The authors declare no conflict of interest.

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