Review

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Chromatographic analysis of toxic phosphylated oximes (POX): a brief overview

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Poisoning with organophosphorus compounds (OP), e.g. pesticides and nerve agents, causes inhibition of acetylcholinesterase (AChE) by phosphylation of the active site serine residue. Consequently, accumulation of stimulating acetylcholine in the synaptic cleft induces cholinergic crisis which ultimately may lead to death. For standard causal therapy, enzyme reactivators are administered representing oxime derivatives of quarternary pyridinium compounds, e.g. pralidoxime (2-PAM), obidoxime and HI 6. The mechanism of action includes removal of the phosphyl moiety by a nucleophilic attack of the oximate molecule substituting the enzyme and forming a phosphylated oxime (POX). POX is produced in stoichiometric amounts of reactivated enzyme and exhibits a significantly enhanced toxicity (inhibition rate constant) when compared to the parent OP. However, stability of POX under physiological conditions appears to be highly limited. Nevertheless, the presence of POX reveals a potential critical issue for both therapeutic efficacy *in vivo* and pharmacokinetic and pharmacodynamic (PK-PD) modelling based on cholinesterase activity data. Detailed characterization represents an important need for elaboration of the entire oxime pharmacology.

Nevertheless, reports on POX toxicity and analysis are quite rare and may therefore be indicative of the challenge of POX analysis. This review provides a concise overview of chromatographic approaches applied to POX separation. Chromatography represents the key technology for POX purification and quantification in kinetic *in vitro* studies using buffers and biological fluids. Applications based on reversed-phase chromatography (RPC), ion pair chromatography (IPC) and an affinity approach as well as thin layer chromatography (TLC) are discussed and novel applications and data are presented. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: enzyme reactivation; nerve agents; organophosphorus compounds; pesticide; phosphylated oxime; oxime; chromatography

Introduction

Organophosphosphorus compounds (OP), including pesticides and nerve agents, are highly potent inhibitors of cholinesterases (ChE), e.g. acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8; Figure 1).[1] AChE plays a key role in inactivating (hydrolyzing) the neurotransmitter acetylcholine in the central and peripheral nervous system of vertebrates.[2,3] Irreversible inhibition of AChE is caused by phosphylation of the active site serine residue (Figure 2) inducing cholinergic crisis which ultimately may lead to death. In this context, phosphylation denotes both phosphorylation (inhibition by organophosphates) as well as phosphonylation (inhibition by organophosphonates).

Effective standard treatment of OP poisoning includes administration of atropine for symptomatic therapy (antagonizing muscarinic receptors) and oximes for causal therapy.[4,5] Oximes restore AChE activity and initiate recovery of impaired body functions (Figure 2). The most important therapeutic oximes comprise pralidoxime (2-PAM) and obidoxime, which are in clinical use in different countries, and HI 6, that is currently under clinical development[5] (Figure 1).

Therapeutic oximes are positively charged quaternary N-alkylated pyridinium salts comprising either one pyridinium (monopyridinium oxime) or two pyridinium rings (bispyridinium oxime) that are linked by a molecular bridge (e.g. $-CH_2-O-CH_2-$). The aromatic ring contains an oxime moiety (-CH=N-OH) located either in 2- or 4-position and may be additionally substituted by an amide group (Figure 1).

Reactivation of inhibited ChE follows a nucleophilic attack of the oximate at the phosphorus atom (S_N2) thus generating an intermediate complex comprising of oxime, ChE and OP. Hydrolysis of this intermediate leads to a fully reconstituted enzyme, acting as a leaving group. The remaining molecule represents the OP moiety coupled to the oxime and is referred as phosphylated oxime (POX) (Figure 2).[5–10] Under physiological conditions, POX can either be degraded non-enzymatically,[11,12] hydrolyzed enzymatically by plasma enzymes,[1,13,14] or it may re-inhibit ChE (reverse reaction of reactivation)[15] as illustrated in Figure 2. The latter inhibitory reaction results in a diminished net reactivation of AChE and may thus potentially affect the efficacy of oxime therapy.

POXs were shown to be markedly more potent inhibitors of AChE than the parent OPs.[14,16–18] Kiderlen *et al*. compared the inhibition rate constants (k_i) of paraoxon and its corresponding product diethylphosphoryl-obidoxime (DEP-obidoxime; Figure 1) towards human AChE and determined a 350-fold higher k_i for POX.[14] Accordingly, it was found that the k_i-value of diisopropylphosphoryl-obidoxime (DIP-obidoxime; Figure 1) was 600-fold higher than that of diisopropylfluorophosphate (DFP).[17]

In addition, the chemical structure of POX (position of oxime group) determines hydrolytic stability. Ashani *et al*. demonstrated that non-enzymatic degradation of phosphylated 2-PAM

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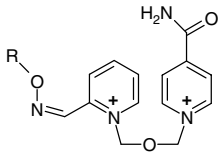
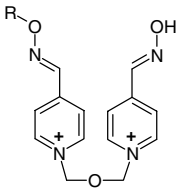


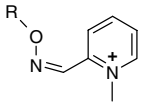
R = H
HI 6

R =
EMP[18]



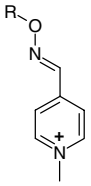
R = H
obidoxime

R =
DEP[14, 17]
DIP[17]
DMP[17]
EMP[16, 18]
i-BMP[16]
n-BMP[16]



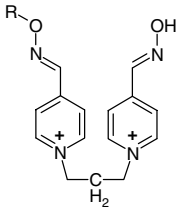
R = H
2-PAM
(pralidoxime)

R =
CMP[19]
EMP[19]
IMP[19]



R = H
4-PAM

R =
CMP[19]
EMP[19]
IMP[19]



R = H
TMB4

R =
EMP[18]

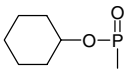
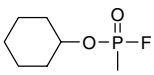
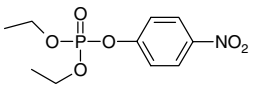
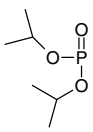
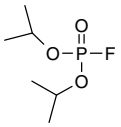
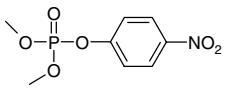
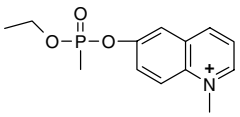
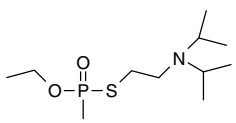
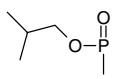
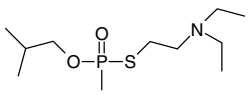
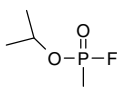
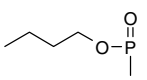
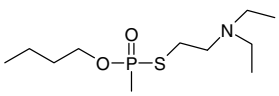
R =	phosphyl moiety		parent OP	OP structure
CMP	O-cyclohexyl methl phosphonyl		cyclosarin (GF)	
DEP	diethyl phosphoryl		paraoxon-ethyl	
DIP	diisopropyl phosphoryl		diisopropylfluoro-phosphate (DFP)	
DMP	dimethyl phosphoryl		paraoxon-methyl	
EMP	O-ethyl methyl phosphonyl		7-(methylethoxy phosphinyl oxy)-1-methyl quinolinium (MEPQ)	
			VX	
i-BMP	O-isobutyl methyl phosphonyl		Russian VX (VR)	
IMP	O-isopropyl methyl phosphonyl		sarin (GB)	
n-BMP	O-n-butyl methyl phosphonyl		Chinese VX (CVX)	

Figure 1. Chemical structures of therapeutic oximes, their phosphylated products and corresponding organophosphorus compounds. Numbers in brackets refer to the reference number.

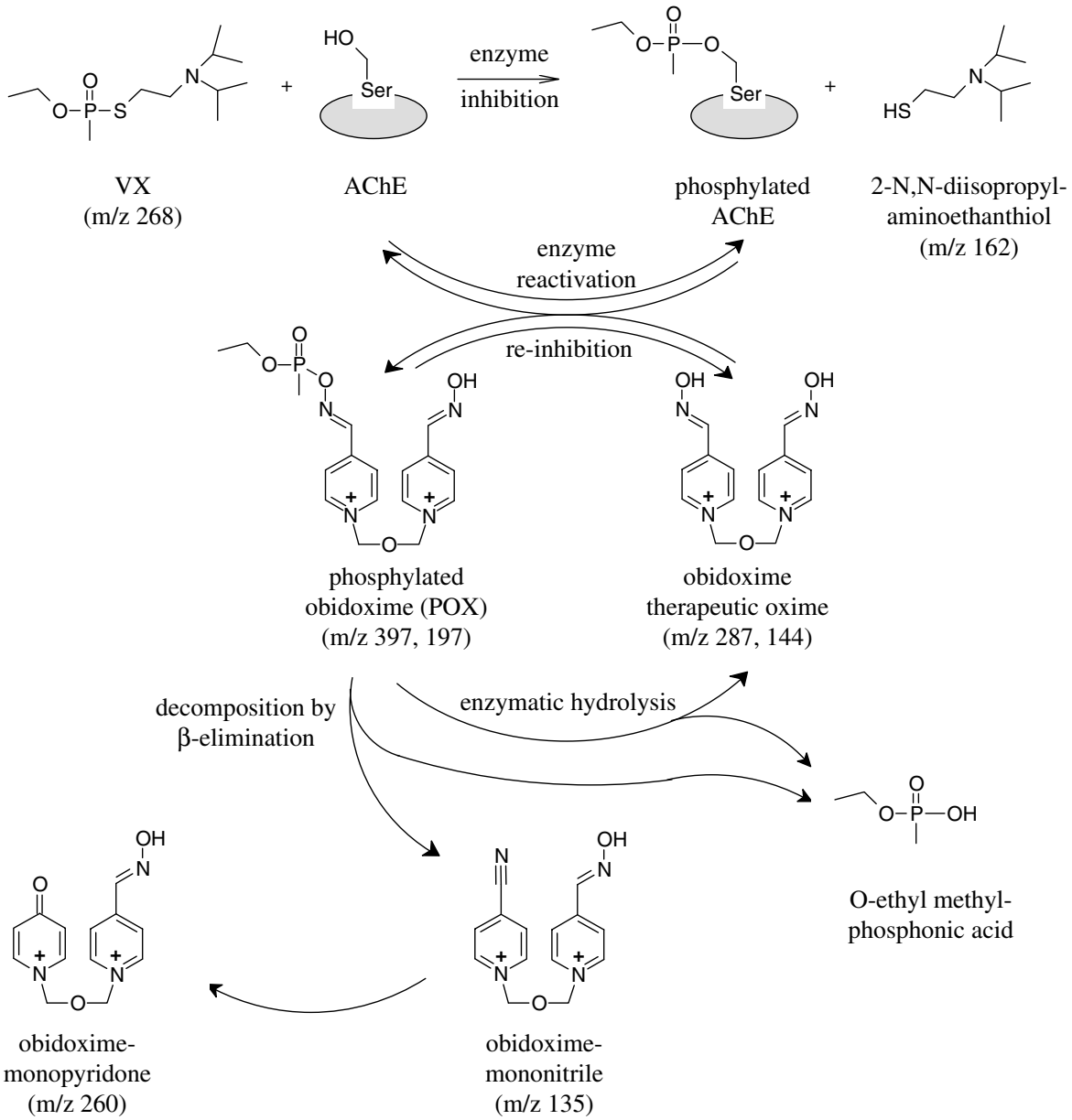


Figure 2. Fate of phosphylated obioxime during inhibition and reactivation of acetylcholinesterase. AChE, acetylcholinesterase. Structures and *m/z* values are derived from LC-ESI-MS analysis according to Becker[16].

happened almost two orders of magnitude faster than that of 4-PAM and three orders of magnitude faster than that of 3-PAM (Figure 1).[19]

Therefore, detailed data on POX stability, toxicity, and the effect on net reactivation in oxime therapy are of great pharmacological importance. Nevertheless, so far, few research groups have dealt with synthesis, analysis, and chemical and pharmacological characterization of POXs *in vitro*.

Chromatography has been shown to be an essential tool for preparative purification and determination of purity after synthesis. In addition, analytical separation techniques were successfully used to monitor concentration-time profiles of POX in physiological buffers and biological fluids. This review provides an overview on these activities and – to the best of our knowledge – presents a limited but complete summary of chromatographic techniques introduced so far.

Synthesis, Spectroscopic Characteristics and Stability of Phosphylated Oximes

This section provides essential background information on the production and characterization of POX that is essential for understanding the chemical and toxicological properties and relevant for UV-detection and determination of enzymatic and non-enzymatic degradation kinetics.

Synthesis

Phosphylated derivatives of pralidoxime were usually synthesized in two steps using monopyridine oxime as educt not methylated at the N-atom. This compound was allowed to react directly with an excess of OP to phosphylate the oxime moiety. Residual OP was removed by liquid-liquid extraction (LLE) with an organic

solvent (e.g. $CHCl_3$) sometimes followed by gravity column chromatography (straight phase on silica). In a second step, the tertiary POXs were converted with methylating agents (e.g. MeI) to achieve quaternization at the N-atom yielding in a single charged cation. This POX salt precipitated thus enabling product purification. Following this two-step strategy, pure products were obtained in moderate yields of about 30–57%.[19–26]

However, this strategy was not transferable to the synthesis and purification of phosphylated bispyridinium derivatives due to the lack of tertiary precursors containing a linker connecting the N-atoms of both pyridinium rings (Figure 2). Therefore, direct reaction of intact quarternary bicyclic oximes with OP was carried out, thus requiring subsequent chromatographic purification instead of crystallization (Figure 1).[14,17,18,24,25] Following this approach, for example, Waser *et al*. produced mono- and di-phosphonylated obidoxime from direct reaction with sarin[27] and Becker reported on POX formation from obidoxime and V-agents.[16]

Subsequent structural identification of POXs was typically carried out by electrospray ionization mass spectrometry (ESI-MS/MS) targeting molecular weight and fragmentation pattern as well as by nuclear magnetic resonance spectroscopy (NMR) including ^{1}H- and ^{31}P-NMR.[14,16,18]

Spectroscopic characteristics

Due to the delocalized Π-electron system of the pyridinium ring and its oxime moiety, corresponding compounds exhibit strong UV-absorption maxima (λ_{max}) at about 280 nm. In addition, absorbance depends on pH-controlled oxime dissociation (deprotonation) to its zwitterionic oximate betaine structure causing a bathochromic shift to an additional UV-absorbance maximum at e.g. 353 nm for deprotonated obidoxime.[14,27]

Phosphylation of the oxime group causes characteristic electronic alterations and therefore a hypsochromic shift of λ_{max} to shorter wavelengths.[11,12,14,17,26,27] These changes were often used to monitor formation and decomposition of POX although the difference between both maxima was often quite small ($\Delta\lambda \approx 10$ nm). Whereas λ_{max} of non-phosphylated obidoxime was found at 286 nm (pH 4–5, $\varepsilon = 34.5\ mM^{-1}\ cm^{-1}$)[16], its corresponding POX-derivatives showed absorption maxima at, for example, 273 nm (DEP-obidoxime, $\varepsilon = 31.6\ mM^{-1}\ cm^{-1}$),[14] 275 nm (EMP-obidoxime, O-ethyl-methylphosphonyl-obidoxime, $\varepsilon = 27.5\ mM^{-1}\ cm^{-1}$ and n-BMP-obidoxime, O-n-butyl-methylphosphonyl-obidoxime, $\varepsilon = 26.9\ mM^{-1}\ cm^{-1}$),[16] and 278 nm (i-PMP-obidoxime, O-isopropyl-methylphosphonyl-obidoxime, ε not determined)[27] (Figure 1).

Accordingly, monitoring UV-spectra of the reaction mixture allowed to analyze the reaction of OP nerve agents (sarin, crotylsarin, cyclosarin, soman, tabun, and VX) with several mono- and bispyridinium oximes in morpholinopropanesulfonic acid buffer (MOPS, pH 7.4) as described by Becker *et al*.[11,12] Corresponding kinetics of formation and decomposition of POX were calculated without further purification and without isolation of POXs. The lack of compound separation might be disadvantageous when unexpected decomposition or byproducts interfere. Furthermore, UV-maxima are not baseline resolved, thus causing overlapping UV-profiles that hamper or even complicate quantitative analysis. Therefore, chromatographic separation of compounds of interest appears to be more appropriate and beneficial than simple cuvette tests.

In general, the differing wavelengths of oximes and POXs are also valuable for analysis by high-performance liquid chromatography (HPLC) to optimize selectivity and sensitivity in UV-detection although not being used consistently in the referred literature as pointed out below.

Decomposition and hydrolysis

Under physiological conditions POXs are quite sensitive towards degradation by enzymatic or non-enzymatic processes that determine the apparent half-life and thereby may effect net reactivation of inhibited AChE during oxime therapy.[13,21,23,28]

Non-enzymatic decomposition of POX yields in a nitrile product by β-elimination followed by monopyridone production (Figure 2). The decrease of POX concentration was quantified by well-established Ellman-based assays monitoring the loss of AChE inhibitory activity.[14,16–18,20,29–32] However, these enzymatic assays provide functional data of the entire reaction mixture but do not allow to follow concentration-time profiles on the molecular level. Selective HPLC procedures overcome this drawback. Therefore, corresponding POX degradation rates depending on diverse solvents and physiological media were elaborated by HPLC-UV.[14,16–18,20,32] The reaction was monitored either by measuring the time-dependent decrease of the POX peak or the increase of the nitrile peak. Kiderlen *et al*. found the apparent half-life of DEP-obidoxime (Figure 1) to be 13.5 min in buffer (pH 7.1) at 37 °C.[14] The relevant methods will be discussed below in detail.

Enzymatic hydrolysis of POX in physiological systems, like plasma, is known to be catalyzed by, for example, paraoxonase (PON1, EC 3.1.8.1, formerly EC 3.1.1.2) and POX-hydrolase. Enzymatic cleavage leads to oxime reconstitution and formation of the corresponding phosphoric or phosphonic acid thereby detoxifying the organism (Figure 2).[13,14,17,28]

PON1 is a calcium-dependent liver-expressed P450 phosphotriesterase belonging to the class of A-esterases with broad substrate specificity towards various lactones, esters, and toxic products produced by lipid oxidation. This enzyme is also known to hydrolyze OP.[33,34] Two homocygote phenotypes of PON1 were described exhibiting glutamine/arginine (Q/R) point mutation at position 192: $PON1_{192R}$ and $PON1_{192Q}$.[35,36] Levels and genetic variability of PON1 influence sensitivity and substrate specificity.[34,35]

Kiderlen *et al*. described a Ca^{2+}-dependent POX-hydrolyzing enzyme activity that is present in human plasma to an individual extent.[14] They concluded that this POX-hydrolase represents a phenotype of PON1. Nevertheless, experimental evidence on the molecular level based on, for example, mass spectrometric or amino acid sequence analysis was not provided.

However, therapeutic detoxification catalyzed by PON1 variants might be an important step in modulating toxicokinetic behaviour of OP and POX during oxime therapy. To elaborate this effect on the molecular level several procedures for POX quantification were required which measure the corresponding hydrolysis products, for example, nitriles and oximes.[14,17] For this purpose, liquid chromatography proved to be a very useful tool to monitor POX concentrations in *in vitro* samples.[17,22]

Chromatographic Analysis of Phosphylated Oximes

Generally, reversed-phase material was used for the analysis and isolation of POX. Under acidic conditions POXs derived

Table 1. Column chromatographic methods for analysis of phosphylated oximes (POX). ACN, acetonitrile; DEP, diethyl phosphoryl; DIP, diisopropyl phosphoryl; DMP, dimethyl phosphoryl; EMP, O-ethyl methyl phosphonyl; +ESI, electrospray ionization in positive mode; HOAc, acetic acid; i-BMP, O-isobutyl methyl phosphonyl; L : length, I.D., inner diameter; MeOH, methanol; n-BMP, O-n-butyl methyl phosphonyl; NH_4FA, ammonium formate; NH_4OAc, ammonium acetate; PIC A and PIC B7, ion-pairing reagents (Waters, Eschborn, Germany)

stationary phase	dimension L × I.D [mm × mm]; particle size [µm]	mobile phase solvent A solvent B	flow [mL/min]	detection [nm]	POX	ref
Atlantis dC18 T3	150 × 4.6 5	A: 100 mM NH_4FA in H_2O B: 100 mM NH_4FA in MeOH/H_2O 50 : 50 gradient mode	1.0	275; 286; 354	i-BMP-obidoxime n-BMP-obidoxime EMP-obidoxime	16
	15 × 2.1 5	A: 100 mM NH_4FA in H_2O B: 100 mM NH_4FA in MeOH/H_2O 50 : 50 gradient mode	0.208	+ESI-MS	i-BMP-obidoxime n-BMP-obidoxime EMP-obidoxime	16
LiChroCart Supersphere 60, RP-select B	125 × 3 4	A: 10 mM NH_4OAc (pH 4.5) in H_2O B: 10 mM NH_4OAc (pH 4.5) in MeOH/H_2O 50 : 50 gradient mode	0.6	260; 300 off-line +ESI-MS 220; 285	DEP-obidoxime DIP-obidoxime DMP-obidoxime	14 17
	125 × 4 5	A: 4% v/v PIC B7®, 0.35% v/v PIC A® in H_2O B: ACN gradient mode	1.2	260; 300	DEP-obidoxime	14
	125 × 3 4	4% v/v PIC B7®, 0.35% v/v PIC A® in ACN/H_2O 12 : 88 or 16 : 84 or 6 : 94 isocratic	0.8	220; 285	DEP-obidoxime DIP-obidoxime DMP-obidoxime	17
Vydac narrow bore C18	250 × 2.1 5	5 mM 1-heptanesulfonic acid in ACN/HOAc/H_2O 20 : 0.5 : 79.5 (pH 3.8) isocratic	0.3	284; 354	EMP-HI-6 EMP-obidoxime	18
µBondapak C18	300 × 3 9	5 mM 1-heptanesulfonic acid in ACN/HOAc/H_2O 20 : 0.5 : 79.5 (pH 3.8) isocratic	0.3	284; 354	EMP-HI-6 EMP-obidoxime EMP-TMB4	18

from quarternary oximes are positively charged compounds. For analyte homogeneity, the acidic pH of the mobile phase has to be strictly controlled favouring the non-dissociated form of the oxime group.[27] Otherwise diffuse retention behaviour or peak broadening will prevent satisfying chromatographic resolution and separation.

Therefore, modifications of the mobile phase were applied including the use of different organic modifiers, buffer concentrations or the addition of negatively charged ion-pairing reagents, e.g. heptanesulfonic acid.[37]

Interestingly, neither cation ion exchange (CEC) nor anion exchange (AEC) chromatography has been reported to be used for POX separation despite the properties of charged molecules. This might be due, for example, to (1) the incompatibility of solvents (salt buffers) with MS detection; (2) the expected broad peaks typical for ion exchange chromatography; (3) unfavourable pH conditions for binding to and desorption from the stationary phase; or (4) insufficient resolution of POX and its educts and byproducts.

However, other approaches making use of column chromatography are presented and discussed below and summarized in Table 1.

Reversed-phase chromatography

For isolation of DEP-obidoxime, which was synthesized from paraoxon and obidoxime (Figure 1), a semi-preparative reversed-phase chromatography (RPC) (LiChroCart Supersphere 60, RP select B 125 mm × 3 mm I.D., 4 µm, Merck) was applied using buffer A (10 mM NH_4OAc in water, pH 4.5) and a linearly rising portion of buffer B (10 mM NH_4OAc in water/MeOH 50 : 50 v/v) (Table 1).[14] No additional ion pair reagent typical for oxime separation was used. The most polar compound obidoxime was eluted first, followed by DEP-obidoxime. Paraoxon as the most lipophilic ingredient of the reaction mixture exhibited the longest retention time. Although DEP-obidoxime exhibited its UV-absorption maximum at 273 nm,[14] chromatography was monitored at 260 and 300 nm prior to fractionation. However, although not using the optimum wavelength, detection of POX was sufficient allowing its isolation. Due to the compatibility of the HPLC eluent, fractions could be analyzed directly by infusion ESI-MS for substance identification.

Stenzel *et al.* chromatographically isolated dimethylphosphoryl-obidoxime (DMP-obidoxime), DEP-obidoxime, and DIP-obidoxime which were synthesized by direct reaction of the oxime with paraoxon-methyl, paraoxon-ethyl and DFP (Figure 1)

in 10 mM sodium veronal buffer at pH 7.5 (Table 1).[17] POXs were separated from educts on a semi-preparative column (LiChroCart Supersphere 60, RP-select B; 125 mm × 3 mm I.D., 4 µm, Merck) by gradient reversed-phase HPLC. Eluents containing NH_4OAc were identical to those applied by Kiderlen *et al*. as described above.[14] Peaks were detected by a diode-array detector at 220 nm for OP and 285 nm for oximes and POXs. Collected fractions were evaporated and reconstituted in acetic acid. This method enabled Stenzel *et al*. to separate the different POXs from other compounds and to collect pure material that was used for further characterization of inhibitory potency and hydrolytic stability.

Becker focused on the generation of purified POXs derived from obidoxime and the nerve agents VX, Russian VX (VR) and Chinese VX (CVX) (Figure 1; Table 1).[16] A reversed-phase column (Atlantis dC18 T3, 150 mm × 4.6 mm I.D., 5 µm, Waters) also enabling polar interactions with the analytes was used for semi-preparative separation of the reaction mixture. The mobile phase of the binary gradient system comprised of ammonium formate (NH_4FA) as a volatile buffer without any additional ion-pairing reagents, thus allowing subsequent mass spectrometric detection. Optimal chromatographic separation and resolution was achieved for obidoxime isomers, POX products and byproducts using 100 mM NH_4FA in water (solvent A) and in MeOH/water 50 : 50 v/v (solvent B). This chromatographic setting enabled the separation of EMP-obidoxime (derived from VX), O-isobutyl-methylphosphonyl-obidoxime (i-BMP-obidoxime, derived from VR), and n-BMP-obidoxime (derived from CVX) (Figure 1). Figure 3A depicts a representative chromatogram monitored at 286 nm of a reaction mixture (obidoxime and VX) for EMP-obidoxime synthesis.[16] Interestingly, the stationary phase (Atlantis dC18) allowed to separate two isomers of obidoxime resulting from E/Z-isomerism of both oxime groups. According to Spöhrer and Eyer[38] the most prominent HPLC peak (t_R 5.8 min) was assigned to the syn-syn-isomer (E/E) as illustrated in Figure 1. The much smaller peak (t_R 5.2 min) represents the less abundant (5%) syn-anti-isomer (E/Z). However, no peak splitting occurred for the EMP-obidoxime product (t_R 15.0 min), which might be due to either insufficient chromatographic resolution or to geometrical preferences for phosphylation caused, for example, by steric hindrance. However, the chromatogram demonstrates efficient separation of the POX analyte and the mobile phase provided compatibility to ESI-MS detection that was beneficial for compound identification (molecular weight) and elucidation of structure (Figure 2).

Furthermore, kinetics of POXs formation and degradation were analyzed by HPLC-UV.[16] Production and decomposition of educts, EMP-obidoxime, and degradation products was followed by determination of peak areas (Figure 3B). As molar absorptivities (ε) of these substances are not identical, the areas did not allow exact correlation to molar ratios. Nevertheless, the presented area-time profiles are typical for consecutive reactions illustrating simultaneous product formation and decomposition. It was clearly shown that EMP-obidoxime reached its maximum concentration after 80 min, whereas the amount of mononitrile (decomposition product) increased constantly approaching its maximum close to 300 min.

The modern stationary phase applied represents the key issue of this method by enabling good chromatographic separation combined with MS compatibility.[16] This modified hydrophilic RP material is highly suitable for retention of polar basic and acidic compounds as also demonstrated recently, for example, for basic tropane alkaloids,[39] polar pesticides,[40] or acidic nerve agent metabolites and adducts.[41]

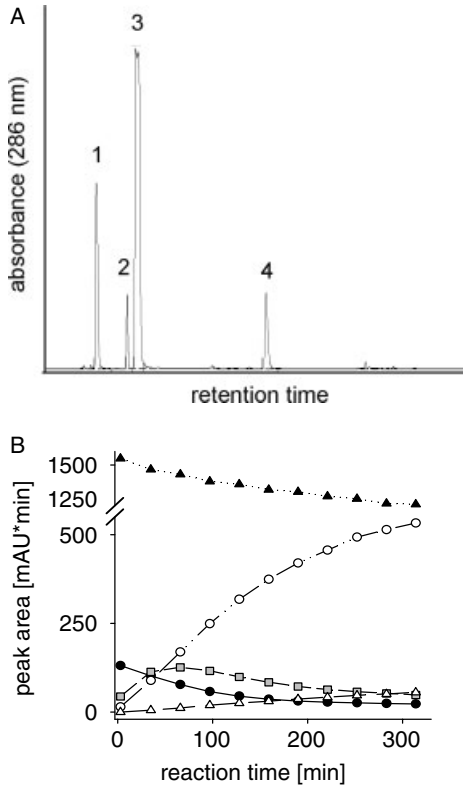


Figure 3. Use of reversed-phase chromatography (RPC) for reaction monitoring of POX synthesis. (A) Representative chromatogram from HPLC-UV analysis of EMP-obidoxime synthesis. Obidoxime and VX were mixed in equimolarity (2 mM in water) and allowed to react for 80 min at 23 °C. Sample was analyzed by RPC (Atlantis dC18) according to the method by Becker referred to in Table 1.[16] (1) t_R 3.0 min, obidoxime-mononitrile; (2) t_R 5.2 min, syn-anti (E/Z) obidoxime; (3) t_R 5.8 min, syn-syn (E/E) obidoxime; (4) t_R 15.0 min, O-ethyl methylphosphyl-obidoxime (EMP-obidoxime). Geometrical isomers were assigned according to Spöhrer and Eyer.[38] Following LC-ESI-MS analysis additional educts and products were found not detectable at 286 nm: t_R 24.4 min, 2-N,N-diisopropylaminoethanethiol; t_R 25.3 min, VX.[16] (B) Educts and products during synthesis of EMP-obidoxime from obidoxime and VX. Obidoxime and VX were allowed to react as described above. Samples were taken at distinct time points and analyzed by the corresponding RPC-UV method. Although peak areas do not exactly correlate to molar ratios of detected compounds, time-dependent courses are typical for a consecutive reaction demonstrating formation of EMP-obidoxime (from obidoxime and VX) and its decomposition into obidoxime-mononitrile and subsequently into obidoxime-monopyridone.[16] Black triangle, syn-syn-(E/E)-obidoxime; white circle, obidoxime-mononitrile; black circle, syn-anti (E/Z)-obidoxime; grey square, EMP-obidoxime; white triangles, obidoxime-monopyridone.

As presented above, RPC is a valuable technique for separating POX from its educts and byproducts useful for preparation and analytical investigations. Despite the positive charge of highly polar quaternary POX, the use of volatile buffers and modern stationary phases enabled sufficient selectivity and peak shape. An alternative modification to overcome the influence of the positive charge is the addition of ion pair reagents resulting in a modified RPC, called ion pair chromatography.

Ion pair chromatography

Ion pair chromatography (IPC) is a method of improving the separation of charged analytes by enhancing peak shape and retention. As the positive charge in the pyridinium ring of the oximes and POX cannot be suppressed by adjusting the pH

as commonly done for dissociating compounds with basic or acidic residues, ion-pairing salts were added to the mobile phase thus modulating hydrophilicity of the analyte complex. Typically, alkylsulfonates were used to retard cationic substances. On the one hand the negatively charged sulfonate moiety interacts with the analyte cation; on the other hand the hydrophobic alkyl chain strongly interacts with the RP material of the stationary phase.

Heptanesulfonic acid is a common ion-pairing reagent that is often applied as a ready-to-use, low-UV-absorbing formulation mixed with additional buffer additives (methanol, acetic acid) provided under the trade name PIC B7® (Waters, Eschborn, Germany). Sometimes PIC B7® is utilized together with PIC A® (Waters, Eschborn, Germany) that contains tetra-alkyl ammonium compounds designed for ionic interactions with anionic analytes. For analysis of therapeutic oximes from pharmaceutical preparations or biological fluids, such IPC-UV approaches very often represent the method of choice.[42] However, the presence of such ion-pair salts typically prevents direct MS detection due to polluting and clotting of the interface and suppression of analyte ionization.

Isocratic separation of oximes and their corresponding POXs, which were synthesized by direct reaction of obidoxime, TMB4 and HI 6 with MEPQ (7-methylethoxy phosphinyloxy-1-methylquinolinium iodide), was achieved using heptanesulfonic acid (5 mM in 20% v/v ACN, 0.5% v/v acetic acid, pH 3.8).[18] This mobile phase was successfully applied for semi-preparative purification on two different C18-columns: Vydac narrow bore (250 mm × 2.1 mm I.D., 5 µm) and µ-Bondapak (300 mm × 3.9 mm I.D., 5 µm, Waters). Peaks were monitored by an UV-detector at 284 and 354 nm enabling detection of maximum sensitivity for the oxime (Table 1). POX containing fractions were collected, pooled, lyophilized, and re-dissolved in D_2O to be used for ^{31}P-NMR measurements allowing elucidation of chemical structures.

Whereas the preceding examples describe the use of IPC for synthesis reaction monitoring and purification of POXs, the following chromatographic approaches were applied to the determination of POX and its degradation products in plasma in *in vitro* stability studies and enzyme assays.[14,17]

Kiderlen *et al*. reported on the quantification of DEP-obidoxime by means of IPC in combination with an enzymatic preparation step carried out by incubation of POX in plasma.[14] Human plasma exhibits POX-hydrolase activity that irreversibly hydrolyses DEP-obidoxime to diethyl-phosphoric acid and obidoxime (Figure 2).[14] Prior to POX addition, plasma was pretreated with soman, a nerve agent that irreversibly inhibits plasma BChE, thereby blocking its active site and averting POX to phosphylate BChE thus preventing false quantitative analysis.

Assuming complete and stoichiometric hydrolysis the amount of liberated obidoxime was a measure of POX thereby allowing its indirect quantification. The oxime was analyzed by isocratic IPC (LiChroCart LiChrosphere 60, RP select B, 125 mm × 4 mm I.D., 5 µm, Merck) using a solvent containing PIC B7® and PIC A® (Table 1). In addition, the use of a binary gradient allowed optimized detection of DEP-obidoxime besides its degradation products obidoxime-mononitrile and obidoxime-monopyridone.[14]

In a comparable enzymatic assay, Stenzel *et al*. quantified DEP-, DIP- and DMP-obidoxime indirectly after enzymatic cleavage of POXs catalyzed by purified PON1.[17] For this purpose, obidoxime was measured by IPC (LiChroCart Supersphere 60, RP select B, 125 mm × 3 mm I.D, 4 µm, Merck) at 285 nm using aqueous PIC B7® and PIC A® containing solvents mixed with ACN as organic modifier.

Stenzel *et al*. also succeeded in the determination of POX-hydrolase activity in crude human plasma by quantifying POXs directly instead of analyzing the hydrolysis products.[17] Three different POX-solutions (DMP-, DEP-, and DIP-obidoxime) were mixed with plasma. These samples were precipitated after distinct incubation periods by the addition of trichloracetic acid, followed by centrifugation. The supernatant was mixed with PIC B7®/NH_3 prior to isocratic IPC on LiChroCart Supersphere 60. For chromatographic separation of the three different POXs, mobile phases containing identical concentrations of PIC B7® and PIC A® with varying concentrations of ACN were applied (Table 1). The need for rising portions of the organic modifier was caused by the differing organic substituents of the phosphyl moiety determining polarity (Table 1).

Time-dependent concentration decay of POX was measured as an indicator for POX-hydrolase activity in human plasma thus proving striking individual differences in enzyme activity which was referred to the presence of the different PON1-phenotypes, $PON1_{192R}$ and $PON1_{192Q}$. The POX-hydrolyzing activity (k_e) of the $PON1_{192Q}$ phenotype in plasma (9.6 min^{-1}) was much stronger than that of the $PON1_{192R}$ phenotype (0.12 min^{-1}).

None of the RP- and IP- chromatographic methods described above was used for absolute quantification of POX so far and consequently no procedure has been validated for this use. In contrast, validated procedures for the measurement of related oximes are used in clinical trials and for pharmacokinetic studies underlining the reliability of such methods.[42–45]

However, the presented approaches were suitable for separation of POXs either from their synthesis educts or their hydrolysis products. Retention on reversed-phase material was determined by individual polarities and charge of the analytes. In contrast, the following chromatographic approach profits from a highly selective affinity step used for POX synthesis and subsequent partial purification.

Affinity chromatography

The procedure described below represents a smart experimental design of on-column affinity adsorption defining a reactor for POX synthesis and allowing selective purification.

Luo *et al*. synthesized POXs by oxime-mediated reactivation of OP-inhibited AChE.[18] AChE from fetal bovine serum was bound to a resin providing procainamide as selective affinity ligand immobilized on sepharose 4B gel. Adsorbed AChE was subsequently treated with a solution of MEPQ in MOPS-buffer (pH 7.4) to phosphylate the active site serine thus inhibiting the enzyme. Following a washing step to remove residual MEPQ, reactivation of AChE was induced by 1 mM obidoxime, TMB4 or HI 6, in the presence of 0.5 mM edrophonium. The latter compound, which is a reversible inhibitor of AChE, was added to prevent the liberated POX from re-inhibition of the free enzyme thus helping to optimize the yield.

Subsequent characterization and identification of POX was carried out as mentioned above by IPC and ^{31}P-NMR. These data provided experimental evidence that POX was formed during the enzyme reactivation process.

Instead of the aforementioned wide-spread column chromatographic methods, an interesting alternative making use of planar chromatography on silica plates was introduced by Waser *et al*.[27]

Thin layer chromatography

Waser *et al*. investigated the reaction of obidoxime with sarin monitoring the hypsochromic shift of the UV-spectrum (λ_{max} for

obidoxime 284 nm and λ_{max} for O-isopropyl-methylphosphonyl-obidoxime, IMP-obidoxime 278 nm) (Figure 1).[27] For isolation and identification of the products thin layer chromatography (TLC) on silica gel plates with a fluorescence indicator was applied. A solution of NH_4Cl (2% w/v) in methanol was used as solvent. The plate was analyzed by fluorescence quenching at an excitation wavelength of 254 nm using a chromatogram spectrophotometer allowing localization of POX and its educts.

In addition, a functional enzyme assay was applied to detect POX by its BChE inhibiting activity. Following this purpose, solutions containing BChE as enzyme, naphth-1-yl-acetate as substrate and Fast Blue B salt as diazo-dye component were sprayed onto the thin layer silica plate.

Regions of the TLC plate, where non-inhibited active BChE was present, showed ester bond cleavage of the substrate thereby releasing naphtholate, which subsequently underwent diazo-coupling with Fast Blue B salt to produce a visible, purple-coloured dye. In contrast, spots containing sarin, POX, and additional ChE inhibitors inhibited BChE and prevented enzymatic hydrolysis of naphth-1-yl-acetate. Consequently, these areas were not coloured thus indicating compounds with inhibitory activity.

Very similar to the established and valuable techniques of zymography, this chromatographic approach combined space-resolved separation with a functional enzymatic assay thereby providing highly useful analytical information on physicochemical properties as well as on biological activity. In principle, this procedure has been shown to be reliable and modifications for high performance TLC (HPTLC) are still in use to detect OP-pesticides, for example, as recently demonstrated by Akkad and Schwack.[46]

Conclusions

It has been shown that POXs produced during oxime therapy of OP poisoning affect reactivation of inhibited ChE and might thus play a role in the pharmacology of recreation. Depending on the chemical structure, POXs exhibit different inhibitory activity and half-lives *in vitro*. Elaboration of this impact represents a challenge for the fields of molecular pharmacology and toxicology.

The highly limited stability of POX caused by rapid enzymatic hydrolysis and non-enzymatic decomposition in physiological systems represents the most important constriction in analysis. This might be one of the major reasons why no data are available so far describing POX concentrations in *in vivo* studies. Therefore, *in vitro* experiments on POX formation and decay in e.g. plasma or buffers simulating physiological conditions are valuable replacements. However, such test systems are only models not representing the real-life situation in living organisms entirely.

Nevertheless, analysis of POX in physiological media requires reliable procedures for sample preparation not accelerating POX-decay as well as chromatographic separations that provide sufficient selectivity in combination with an acceptable run time of analysis. Chromatographic analytical procedures introduced so far still do not meet the requirements pointed out above in an optimum fashion. A concise use of optimum wavelengths in UV-detection or application of sophisticated online mass spectrometry in combination with fast separating LC on small particles (<2 µm) promise to improve methods substantially. Furthermore, sample preparations that inactivate potential POX-hydrolyzing enzymes immediately after drawing of blood samples combined with rapid extraction of POX under controlled pH-conditions will favour reproducible and reliable analyte recovery.

However, analytical approaches presented so far have allowed generation of a number of highly important data relevant for toxicological and pharmacological research and help to define future challenges.

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