Retention Behavior of Pyridinium Oximes on PFP Stationary Phase in High-Performance Liquid Chromatography

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The chromatographic behavior was studied of a series of potential acetylcholinesterase reactivators, pyridinium oximes, bearing linear aliphatic chains of the length of the aliphatic bridge from 1 to 12 carbon atoms, on a pentfluorophenyl-modified stationary phase. The retention mechanisms and the dependence of the capacity factor on mobile phase composition, aliphatic chain bridge length and calculated log P were evaluated and discussed in detail. The separation of the studied oximes was found to be driven by hydrophobic interactions when a lower content of organic modifier was used in mobile phase; however, the ion-exchange mechanism was the leading one when a large portion of organic modifier was used. In addition, the lipophilicity was found to be a driving mechanism of the separation of oximes bearing a connecting chain of the length of 6–12 carbon atoms, whereas the retention of oximes with shorter connecting chains was significantly influenced by other separation mechanisms such as aromatic or π–π interaction. These results can be useful for the development of new, efficient acetylcholinesterase reactivators.

Introduction

Quaternary pyridinium and bispyridinium oximes belong to group of compounds that are widely used as antidotes against organophosphates (OPs) and organophosphonate poisoning (1–3). OPs irreversibly inhibit cholinesterases such as acetylcholinesterase (EC 3.1.1.7) or butyrylcholinesterase (EC 3.1.1.8) through covalent bonding on the serine in the active site of these enzymes (4). The oximes work as cholinesterase reactivators by removing the organophosphorus compounds from the serine site using the nucleophilic oxime moiety.

The group of clinically used oximes includes monopyridinium oxime pralidoxime in the form of chloride (Protopam, ComboPen and 2-PAM chloride) or mesylate (Contrathion) salts and bispyridinium oxime obidoxime chloride (Toxogonin, Pirrangit and Toxobindin) or dimesylylate (Figure 1A). In the case of OP intoxication, oximes HI-6 (Antiva) and Hlo¨7 might be also tested for their reactivation ability toward OP inhibited cholinesterases (1, 5–8).

The purity of synthesized oximes should be evaluated before in vitro or in vivo testing. Usually, chromatographic methods are developed and applied (9). Thin-layer chromatography is used for synthesis screening (10) and high-performance liquid chromatography (HPLC) is used for the evaluation of the final product or its determination in biological materials, such as plasma (9), brain, liver, lung, kidney (11), urine (12), blood and cerebrospinal fluid (13).

In addition, chromatographic methods have been developed and applied for the estimation of the specific biological properties of the studied compounds, e.g., lipophilicity (14, 15); partitioning coefficients (16) such as the octanol–water partitioning coefficient, which is used as an indicator of the hydrophobicity for the estimation of bio-concentration factors (17); dissociation constants (18); solubility (19); oral absorption in humans (20); or permeation through biomembranes (21), for example, the blood-brain barrier (22). Thus, the knowledge of the chromatographic behavior of pyridinium oximes under various conditions may help to understand their biological properties. The pentfluorophenyl-modified (PFP) stationary phase is a prospective tool for the investigation of the physico-chemical properties of chemical compounds with the potential for medicinal use due to the possibility of investigating a wide range of retention mechanisms (23).

The physico-chemical nature of bispyridinium oximes allows their separation on a PFP stationary phase. They are very hydrophilic compounds with low lipophilicity, which is limiting for their separation on reversed-phase chromatographic columns. In addition, their high hydrophilicity limits their transport through the blood-brain barrier.

This work concerns the investigation of oxime retention behavior on a PFP stationary phase for estimation of their biological properties. The oximes were formerly synthesized as homologues of bis-pyridinium 4-positioned bis-oximes differing in the length of the connecting linkage (C1–C12; Figure 1B). Some have already been used for animal experiments or clinical practice; e.g., methoxime, trimedoxime or oxime K074.

Experimental

Chemicals

The HPLC gradient grade acetonitrile, sodium nitrite (p.a. grade) and trifluoroacetic acid (p.a. grade) were obtained from Merck (Darmstadt, Germany). Oxime homologues (methoxime, K191, trimedoxime, K074, K305, K194, K309, K197, K310, K338, K339 and K340) were synthesized and purified at the Department of Toxicology, Faculty of Military Health, University of Defence in Hradec Králové (Czech Republic). The structures of the studied oximes are presented in Figure 2. Water used for preparation
Acetonitrile (ACN) in the mobile phase. Experimental conditions: Kinetex PFP column (2.1 mm; 2.6 μm) from Thermo Scientific, San Jose, CA. The analyses were performed on Xcalibur version 2.5.0 software equipped with an ion trap analyzer. The data collection and evaluation was performed on Xcalibur version 2.5.0 software and an LCQ Fleet mass spectrometer equipped with a photodiode array detector (PDA) and an LCQ Fleet mass spectrometer. The liquid chromatograph Thermo Surveyor Plus consisted of a Spectrophotometric Surveyor Plus, a quaternary pump with a Surveyor Instrumentation Chromservis (Prague, Czech Republic).

The retention data were obtained for all oximes under the reported conditions, but not all were complete due to the very high retention of compounds bearing longer aliphatic connecting bridges between the pyridinium rings. This partial non-completeness of the data had no significant influence on the calculated results. The dependence of the capacity factor on the acetonitrile content varied between 5 and 70% (v/v); specifically, 5, 10, 20, 30, 40, 50, 60 and 70% (v/v). The flow rate was set to 250 μL/min. All 12 oximes were injected as a mixture in a volume of 0.1 μL and concentration of 1 mg/mL. The dead time and dead volume of the chromatographic system were determined using potassium nitrite solution at 1.16 min (290 μL each). The mass spectrometer was operated in single ion monitoring mode and the analytes were detected as twice positively charged ions (m/z 129, 136, 143, 150, 157, 164, 171, 178, 185, 192, 199 and 206) or nitrite ion as a negatively mono-charged ion (m/z 62).

The detection was performed under following conditions: electrospray ionization; capillary voltage, 7 kV; capillary temperature, 200 °C; sheath gas flow rate, 50 arbitrary units (au); auxiliary gas flow rate, 15 au; sweep gas flow rate, 0 au.

Results

The retention data were obtained for all oximes under the reported conditions, but not all were complete due to the very high retention of compounds bearing longer aliphatic connecting bridges between the pyridinium rings. This partial non-completeness of the data had no significant influence on the calculated results. The dependence of the capacity factor on the acetonitrile content in the mobile phase using the Kinetex PFP column is shown in Figure 2. The experimental data were fitted by quadratic regression Eq. 1:

$$
\log k' = A \times \varphi^2 + B \times \varphi + C
$$

where \( \log k' \) is the logarithm of the capacity factor of each oxime; \( A, B \) and \( C \) are the regression coefficients; and \( \varphi \) is the percentage of acetonitrile content in the mobile phase. The coefficients of regression equations are presented in Table I. The U-shaped dependences of \( \log k' \) of separated compounds were registered, except methoxime, on the percentage of acetonitrile in the mobile phase. A continuous decrease of \( \log k' \) was observed for methoxime when the acetonitrile concentration was lowered in the mobile phase.
Also, the dependence of capacity factor on the number of carbons in the connecting chain between both pyridinium rings was determined. It can be expressed by the quadratic equation and (Eq. 2) linear regression model (Eq. 3). The general equations were as follows:

$$\log k' = a \times nC^2 + b \times nC + c$$  

(2)

respectively

$$\log k' = p \times nC + q$$  

(3)

where \(a, b, c, p\) and \(q\) are regression coefficients and \(nC\) is number of carbon atoms in the connecting bridge. The results are presented in Figure 3 and the regression equations are shown in Table II.

When both regression models, linear and quadratic, were compared, the linear model was adequate only in extreme conditions (5, 10 and 70% of acetonitrile), whereas the intermediate conditions were not satisfactorily covered by this model. On the other hand, the quadratic equation model was able to fully explain the relations, and the regression coefficients of quadratic model were very close to 1.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Regression coefficients ((\times 10,000))</th>
<th>(\mathbf{r}^2)</th>
<th>(\Phi_{\text{max}}) (%)</th>
<th>(\log P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxime</td>
<td>3.203 (-29.591) (-1,050.845) 0.9987</td>
<td>3.2</td>
<td>-1.87</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K191</td>
<td>4.748 (-177.241) 2,798.504 0.9008</td>
<td>18.7</td>
<td>-2.80</td>
<td>[0.0]</td>
</tr>
<tr>
<td>Trimepoxide</td>
<td>6.145 (-316.883) 6,088.435 0.9618</td>
<td>25.8</td>
<td>-3.20</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K074</td>
<td>8.087 (-510.528) 17,359.713 0.9759</td>
<td>37.1</td>
<td>-3.07</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K305</td>
<td>10.275 (-937.291) 31,260.668 0.9537</td>
<td>31.8</td>
<td>-3.60</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K310</td>
<td>8.634 (-720.830) 23,713.930 0.9602</td>
<td>35.8</td>
<td>-3.78</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K194</td>
<td>9.107 (-653.423) 16,282.617 0.9830</td>
<td>38.3</td>
<td>-3.49</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K309</td>
<td>9.860 (-755.273) 20,072.324 0.9273</td>
<td>31.8</td>
<td>-3.60</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K197</td>
<td>7.248 (-538.400) 17,359.713 0.9759</td>
<td>37.1</td>
<td>-3.07</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K310</td>
<td>8.634 (-720.830) 23,713.930 0.9602</td>
<td>41.7</td>
<td>-2.78</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K339</td>
<td>10.275 (-937.291) 31,260.668 0.9537</td>
<td>45.6</td>
<td>-2.49</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K338</td>
<td>8.634 (-482.370) 11,280.175 0.9273</td>
<td>46.9</td>
<td>-2.14</td>
<td>[0.0]</td>
</tr>
<tr>
<td>K340</td>
<td>9.882 (-991.487) 37,443.534 0.9885</td>
<td>50.2</td>
<td>-1.79</td>
<td>[0.0]</td>
</tr>
</tbody>
</table>

*Note: The portions of acetonitrile in the mobile phase for the minimum retention time of a compound \((\Phi_{\text{max}})\) and \(\log P\) were calculated with ACD/Labs PhysChem Suite.

### Table II

<table>
<thead>
<tr>
<th>Acetonitrile (%)</th>
<th>Linear regression coefficients</th>
<th>(\mathbf{r}^2)</th>
<th>Quadratic regression coefficients</th>
<th>(\mathbf{r}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>0.0279</td>
<td>1.2784</td>
<td>0.9823</td>
<td>0.0011</td>
</tr>
<tr>
<td>60</td>
<td>0.0431</td>
<td>0.8086</td>
<td>0.9642</td>
<td>0.0026</td>
</tr>
<tr>
<td>50</td>
<td>0.0619</td>
<td>0.4394</td>
<td>0.9490</td>
<td>0.0046</td>
</tr>
<tr>
<td>40</td>
<td>0.0878</td>
<td>0.1175</td>
<td>0.9201</td>
<td>0.0084</td>
</tr>
<tr>
<td>30</td>
<td>0.1366</td>
<td>-0.2361</td>
<td>0.9021</td>
<td>0.0146</td>
</tr>
<tr>
<td>20</td>
<td>0.1898</td>
<td>-0.4755</td>
<td>0.9108</td>
<td>0.0232</td>
</tr>
<tr>
<td>10</td>
<td>0.2966</td>
<td>-0.4201</td>
<td>0.9053</td>
<td>0.0186</td>
</tr>
</tbody>
</table>

*Note: The regression equations are presented for both linear (Eq. 2) and quadratic (Eq. 3) models.

### Discussion

The reason for this complex retention behavior of the compounds originates from the interaction mechanisms between the oximes and the stationary phase. In comparison with separations performed on reversed-phase C-18 columns, where the separation is based only on the hydrophobic properties of the column, the PFP stationary phase allows more types of interaction mechanisms, such as ionic interaction, hydrogen bonding, dipole–dipole, aromatic and \(\pi-\pi\) interactions, in addition to hydrophobic interactions. The strength of these interactions depends on the composition of the mobile phase.

For these bispyridinium oximes, in addition to the permanently charged pyridinium moiety (or moieties), the oxime functional groups represent the polar part of the molecule. The oxime groups are dissociated under alkaline p\(\text{H}\); thus, equilibrium exists between their non-dissociated and dissociated forms. The total molecular charge is given as a sum of the permanent positive charges of the pyridinium rings, charged or non-charged aldoxime groups, and eventually, the charges of other contributing functional groups. Thus, the total charge of the molecule depends on pH. Also, dissociation constants depend on the geometry of the molecules. The aldoxime group can be presented in two geometrical isomers: syn-isomer (syn) or anti-isomer (anti). When two aldoxime groups are presented in the molecule, there are three conformational possibilities: syn-syn, syn-anti and anti-anti. The dissociation constants of different aldoxime isomers are known to be dependent on their steric properties. For example, the p\(\text{K}_a\) of pralidoxime is approximately 8.0 (25) and the prevailing obidoxime syn-syn isomer has a p\(\text{K}_a\) of 8.0, whereas the syn-anti isomer has p\(\text{K}_a\) of 8.3 and the anti-anti form has a p\(\text{K}_a\) of 8.6 (26). Pyridinium oximes are electrically neutral only in alkaline solutions (p\(\text{H} \gg 6\)), in which the permanent positive charge of the nitrogen atom of the pyridinium rings is compensated by the negative charge of the dissociated oxime group.

The positively charged molecules of pyridinium oximes (for pH lower than 6) interact with the free underivatized silanol groups of the stationary phase, which are dissociated under this pH and allow the ion-exchange separation mechanism (23). Kitagawa and Tsuda (25) estimated the dissociation constant for unreacted silanol groups on the surface of a C18 stationary phase to be 10–4.3 from the relationship between pH and electroosmotic flow velocity in capillary chromatography. This

![Figure 3](https://example.com/figure3.png)
suggests the presence of negatively charged silanol groups when an acidic mobile phase is used. For example, 0.1% of silanol groups are dissociated at pH 2.3 and strongly increase with pH value. Only the ion–ion interaction strongly contributes to the retention of the analyte.

The different retention characteristics of methoxime (K154) in mobile phases containing a low percentage of acetonitrile may be explained by the retention mechanisms; specifically, by the contribution of each one. At high organic phase concentrations, this is the major mechanism contributing to retention of positively charged pyridinium oximes on the PFP stationary phase ion-exchange. Lowering the acetonitrile portion in mobile phase increases the effects of other separation mechanisms, such as hydrophobicity. In particular, the aliphatic linker between two pyridinium moieties plays a significant role in the lipophilicity/hydrophilicity of the compounds in this study. In the case of methoxime, the linker is very short and hydrophobic interaction is very weak in comparison with ionic interaction. Due to this, the curve does not follow the U-shape as for other oximes, but continues in the decreasing trend when a mobile phase is used with a low acetonitrile content. The differences in log \( k' \) between members of this series of bispyridinium oximes are caused by differences in the length of aliphatic chains connecting pyridinium units. The steric effect of the pyridinium units of methoxime does not allow hydrophobic interaction between the methylene bridge and the stationary phase.

Furthermore, the molecules of pyridinium oximes contain one or two pyridinium units, which are the source of \( \pi \)-electrons. Also, the bispyridinium oximes have an additional part that works as a linker between both pyridinium parts. The influence of the \( \pi-\pi \) interaction on analyte retention in chromatographic systems increases with a decreased acetonitrile percentage in the mobile phase, which was also observed previously (28–30). Apparently, the molecules of acetonitrile contain a triple bond between carbon and nitrogen atoms in the nitrile functional group. This nitrile group of acetonitrile competes with the oxime and pyridinium parts of the analyte during the interactions with the phenyl groups of the stationary phase.

For this reason, the interaction is neglected in mobile phases with high levels of acetonitrile and forced in mobile phases without acetonitrile or with low acetonitrile levels.

This is the case with U-shaped dependences of log \( k' \) on the percentage of acetonitrile in the mobile phase; this behavior may also be explained by the competition of retention mechanisms. When high or low concentrations of organic modifier of mobile phase are used, the ion-exchange hydrophobic (with \( \pi-\pi \) interaction) retention mechanism prevails and the contribution of others is suppressed. The slight parabolic trend in 30% acetonitrile can be caused by the sterical properties of the molecules.

The steric effect plays an important role in the case of bispyridinium oximes with longer aliphatic connecting chains. In the case of the highly flexible connecting chain, conformational changes are conceivable. Thus, this conformational flexibility leads to the differences in the retention characteristics of the oximes (Figure 4).

Additionally, the compounds from the outer parts of this series are more hydrophilic than those from the middle (the calculated log \( P \) is lower). Thus, this nonlinear trend in dependence between the number of carbons in the connecting chain and the capacity factor is caused by stronger the lipophilic interaction of central members of studied oxime series and also contributes to the U-shaped retention of the pyridinium oximes.

**Relationship between calculated log \( P \) and measured log \( k' \)**

The calculated log \( P \) and the measured log \( k' \) were compared. The graphical representation of this relation shows U-shaped curves for all tested acetonitrile portions in the mobile phase. This means that there is not only one retention mechanism, but more interactions are involved in the separation on the PFP column, as discussed previously. The oxime homologues with a connecting aliphatic carbon chain with a length of 1–5 atoms show a negative correlation between log \( P \) and log \( k' \); thus, the hydrophobic interaction is minor and the other types of interactions significantly contribute to their retention. In contrast,
there a positive near to linear trend was observed in the relation between log $P$ and log $k'$ for oximes bearing a connecting chain of $6\text{–}12$ carbon atoms, which is caused by the relatively strong hydrophobic interaction of oximes with the stationary phase, compared with other possible retention mechanisms. Also, the flexibility with a longer connecting chain may play a role in these observations.

Conclusion

The chromatographic behavior of bispyridinium oximes bearing alkyl connecting bridges was evaluated on a PFP-based stationary phase. No linear trend was observed in the retention behavior under different acetonitrile contents in the mobile phase. The registered U-shaped retention characteristics are the result of the interactions of different oximes with the stationary phase. In the case of the mobile phase with a low content of acetonitrile, they can be assigned primarily to the hydrophobic interaction between oximes and the PFP stationary phase. When a high percentage of acetonitrile in mobile phase is used, the contribution of ion exchange is dominant. Additionally, hydrogen bonding, dipole–dipole interactions (cation–π) and π–π interactions should be considered. The U-shaped graph of the dependence of the logarithm of the oxime retention factors on the number of the carbon atoms is a result of various strengths of participating separation mechanisms, as a function of the length of a connecting chain, especially its flexibility. The molecules of oximes with connecting chains longer than six carbon atoms are more flexible and primarily undergo hydrophobic interaction with the stationary phase, whereas the retention of these with shorter ones is influenced by other interactions. Also, possible conformational changes in this linking chain play roles in retention. The results can be helpful in understanding the pharmacological properties and the design of new acetylcholinesterase reactivators with targeted properties.

Acknowledgments

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