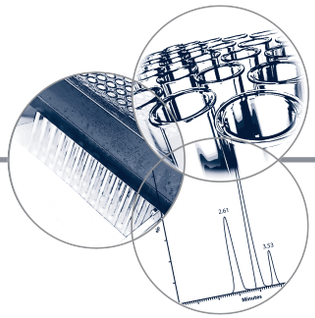
RESEARCH ARTICLE



LC–MS/MS approaches for the assay of *bis*-quaternary pyridinium oximes used as AChE reactivators in biological matrices

Background: Extreme efforts are made for the structural diversification of oximes used as AChE reactivators. Co-administration of different oximes should also be considered as a solution in therapy. Consequently, development of selective assays of oximes in biological matrices is of major importance. **Results:** Three chromatographic separation mechanisms were evaluated: hydrophilic-interaction LC; mixed reversed-phase/cation exchange (DUET); and reversed-phase ion pairing based on per-fluorinated agents. MS was used to identify and quantify oximes. Alternative preparation of whole blood and plasma samples were used based on protein precipitation through addition of acetonitrile or ionic liquids. Quality characteristics of the proposed analytical approaches are discussed. **Conclusion:** The reversed-phase ion pairing based on per-fluorinated agents chromatographic separation mechanism and positive ESI-MS/MS detection produced the best results for the assay of *bis*-quaternary pyridinium oximes. LLOQ in the tenths of nanogram per milliliter range are achievable.

Quaternary ammonium oximes are antidotes used during treatment of poisoning with organophosphorus compounds (OP). Their function consists in reactivation of the inhibited AChE following exposure of humans to OP, arising on battlefields, terrorist attacks, occupational hazards or intakes with suicidal intent. Chemical classification of oximes has been reviewed recently [1].

The therapy with oximes simultaneously depends on the inhibitory potency of the OP compound, the intrinsic toxicity and the reactivating potency of the oxime, the spontaneous reactivation and the aging processes [2,3]. Due to the complexity of the whole scenario, robust analytical tools are needed to support the following study directions: purity control during synthesis of the new oximic congeners; stability concerns on storage of antidotal pharmaceutical formulations; evaluation of the ability of the new candidates to penetrate through biological barriers (especially the blood–brain barrier); evaluation of the efficacy/intrinsic toxicity, either through *in vitro* or *in vivo* screening; and dosing the antidote during the therapy.

Literature data specifically dedicated to analytical issues focused on AChE reactivators is rather modest. UV spectroscopic, chromatographic and electrophoretic methods for the cholinesterase reactivating antidote pralidoxime (PAM) were recently reviewed [4]. Analytical control of purity and stability was discussed for HI-6 [5–7] and obidoxime [8]. Hydrophobicity/hydrophilicity descriptors resulting from the chromatographic behavior of oximes on different stationary phases were proposed [9,10]. The *in vitro* screening of penetration through biological barriers based on the chromatographic retention parameters and correlations with the molecular structure is also available [11,12]. LC–MS/MS approaches have been used for monitoring plasma levels of PAM and other active principles used during poisoning therapy (i.e., atropine and diazepam) [13], the study of blood–brain barrier penetration based on brain microdialysis [14], and partition after administration of therapeutic doses in different parts of the brain, liver, lungs and kidneys [15–17]. LC–diode array detection (DAD) was successfully used for the assay of PAM in urine *in vivo* collected samples in a pesticide poisoning scenario carried out on minipigs [18].

The study of the biological membrane penetration by oximes from chromatographic retention data is generally based on the use of phosphatidyl choline chemically modified aminopropyl silicagel type stationary phase (IAM.PC.DD column, Regis Technologies [IL, USA]) [11,12]. However, hydrophobicity/hydrophilicity descriptors readily to correlate with log K_{ow} (logarithm of the partition coefficient between *n*-octanol and water) may be obtained from the chromatographic retention behavior of oximes on octyl modified silicagel, perfluorophenyl modified silicagel, α1-acid glycoprotein immobilized

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storage (alone or in formulations). Undoubtedly, HILIC is the less suitable chromatographic separation mechanism, considering both selectivity and peak symmetry.

A systematic study of (+)ESI and CID fragmentation of oximes was provided. Although MS/MS can provide the additional required selectivity to support non-ideal chromatographic separation, ion extraction from solutions and transfer of ions towards the mass analyzer are surprisingly low.

Two biological matrices were considered: human whole blood and plasma. Two alternative protein precipitation procedures were used: through addition of acetonitrile or ionic liquids. Ionic liquid addition demonstrated its suitability only for plasma samples. The advantages of protein precipitation by ionic liquid addition refer mainly to less sample dilution and to the possibility of the injection of higher volumes from the supernatant.

Some method validation aspects were discussed. Experimental data indicated the possibility of assaying *bis*-quaternary pyridinium oximes by means of the PF-IPRP chromatographic separation and (+)ESI-MS/MS detection, with LLOQs placed in the tenths of nanogram per milliliter range.

Future perspective

The continuous future diversification of oxime type reactivators and development of the mixed therapies will further encourage the evaluation of selective separation alternatives for resolving closely structurally related congeners. Additional understanding of the MS and MS/MS behavior of oximes, as well as the use of high-resolution mass analyzers (i.e., TOF, orbital traps or ion cyclotron resonance) will act as an additional tool for tuning the selectivity of the whole analytical process. LC–MS/MS approaches will certainly became the preferred tools for solving difficult topics related to oxime-type AChE reactivators: structure development, purity control during synthesis, stability control, toxicity and efficacy studies, dosing during treatment, differential partition in living organisms and assays in biological matrices.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Selective chromatographic mechanisms have been considered for separation of* bis*-quaternary pyridinium oximes

- Hydrophilic-interaction LC, mixed reversed-phase/cation exchange and per-fluorinated ion pair reversed-phase were considered.
- The ion pair reversed-phase mechanism using heptafluorobutyric acid as pairing agent produced the highest selectivity, combined with suitable peak shapes and increased chromatographic efficiency.

A systematic study of MS behavior of* bis*-quaternary pyridinium oximes is presented

- Although the molecular dication is conserved in the ESI source, further fragmentation patterns based on proton or proton/NO· elimination occurs.
- Tentative collisional-induced dissociation fragmentation patterns of the some important precursor ions are presented.

Two alternative preparation approaches for whole blood and plasma samples were evaluated

- A new approach based on addition of an ionic liquid for protein precipitation in human plasma samples was proposed.
- Both protein precipitation approaches (based on acetonitrile or ionic liquid addition) behave similarly for preparation of plasma samples. However, better sensitivity is somehow obtained through the ionic liquid addition approach.

Quality characteristics of the proposed analytical approaches are presented

- Selectivity, matrix effects, linearity, precision and accuracy met criteria widely accepted by bioanalytical guidelines in force.
- LLOQs in the tenths to hundreds of nanogram per milliliter were obtained.