High-Performance Liquid Chromatography Analysis of By-Products and Intermediates Arising During the Synthesis of the Acetylcholinesterase Reactivator HI-6

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Abstract

An high-performance liquid chromatography (HPLC) method for identification of quaternary and non-quaternary compounds (parent compounds, intermediates, by-products, and products) within the synthesis of the acetylcholinesterase reactivator HI-6, the most promising antidote of nerve agent poisonings, is described. This HPLC method could be of high interest as a quick purity control for those who are interested in development of new acetylcholinesterase reactivators as well as for those who are interested in the synthesis of HI-6 in laboratory or in large-scale production. An HPLC method for quaternary compounds without using common ion-pairing reagents was developed, too.

Introduction

At present, there is long lasting threat of possible misuse of chemical warfare agents. Probably the most well-known misuse of these agents is the sarin Tokyo subway attack in 1995. Thanks to the quick administration of nerve agent antidote acetylcholinesterase (AChE; EC 3.1.1.7.) reactivator pralidoxime, only twelve people died and several thousands had mild-to-severe intoxication symptoms (1).

Organophosphorus pesticides are, from a chemical point of view, very close to nerve agent and due to this their toxic affect is also similar in inhibition of cholinesterases in human body and subsequent cholinergic crisis (2). Thanks to the similar mode of toxic action, antidotal treatment is also very similar: anticholinergics and acetylcholinesterase reactivators are generally used (3,4). However, it was found that AChE reactivators are inhibitor-dependent and is not able to treat all nerve agent-caused intoxications due to this one reactivator. It means that no single broad spectrum reactivator exists (5,6). Due to this, there are many efforts to prepare more promising AChE reactivators throughout the world (7,8). These days, oxime HI-6 seems to be the most promising AChE reactivator as substitute of the obsolete pralidoxime and obidoxime (9). Reactivation potency of HI-6 surpassed potency of pralidoxime and obidoxime in almost all nerve agent intoxications. However, in the case of tabun and pesticide poisonings, there is still lack of the optimal AChE reactivator (10,11). Despite its lower potency in tabun and pesticides, HI-6 is number one among the AChE reactivators; and because of this it is under investigation in many armies throughout the world.

Although synthesis of the oxime HI-6 was published and patented several times, preparation of a compound without impurities is still needed (12–14). There is no available summary work dealing with rapid and complex determination of such impurities at the present time. Accessible papers describing assessment of HI-6 impurities are aimed only at the detection of decomposition products of this reactivator during storage of its crystalline form or solutions, usually in the mixture with another therapeutics (atropine, diazepam) (15–17).

To get quick monitoring of the purity of all intermediates, by-products and products within the synthesis of HI-6, we focused our attention on the development of the convenient high-performance liquid chromatography (HPLC) method, which could be applicable in every chemical laboratory. Both preferred HI-6 salts dichloride (6) and dimethanesulphonate (7) were included in this study. Moreover, parent substances 2-hydroxyiminomethylpyri-

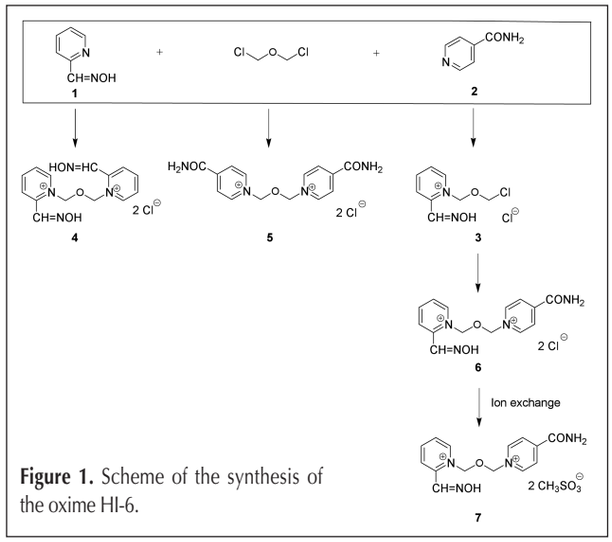

Figure 1. Scheme of the synthesis of the oxime HI-6.

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dine (1) and 4-carbamoylpyridine (2), intermediate (2-hydroxyiminomethyl-1-(chlormethoxymethyl) pyridinium chloride (3), and potential by-products 1,3-bis(2-hydroxyiminomethylpyridinium) oxapropane dichloride (4), and 1,3-bis(4-carbamoylpyridinium) oxapropane dichloride (5) were considered in this study. For better understanding of the selected substances, the common synthetic process is outlined in Figure 1.

Organophosphate antidotes containing quaternary nitrogen atoms in the molecule is necessary to adapt to the technical possibilities of separation in the area of HPLC. For this reason, additives to the mobile phase are used, enabled by the principle of forming ionic pairs with the analyte. A commonly used ion-pairing reagent is octane-1-sulphonic acid sodium salt. This salt binds with its non-polar chain to the stationary phase and creates an alternative environment with its polar part for separation of ionic compounds. Tetramethylammonium chloride is used as a competitory reagent influencing retention time. Ratio of these two additives easily influences the retention as needed. The type of stationary phase RP-C_{18} is not so important in this case.

Table I. Basic Physical Properties of Tested Compounds

No.	Compound	Structure	Mw	UV max (nm)	m.p. (°C)
1	Pyridine-2-aldoxime	$C_6H_6N_2O$	122.12	275	105–107
2	Isonicotinamide	$C_6H_6N_2O$	122.12	267	156–158
3	Monoquaternary P2A	$C_8H_{10}Cl_2N_2O_2$	237.08	307	146–150
4	Bisquaternary INA	$C_{14}H_{16}Cl_2N_4O_3$	359.21	268	234–236
5	Bisquaternary P2A	$C_{14}H_{16}Cl_2N_4O_3$	359.21	306	199–201
6	HI-6 Dichloride	$C_{14}H_{16}Cl_2N_4O_3$	359.21	308	150–152
7	HI-6 Dimethanesulphonate	$C_{16}H_{22}N_4O_9S_2$	478.50	308	173–175

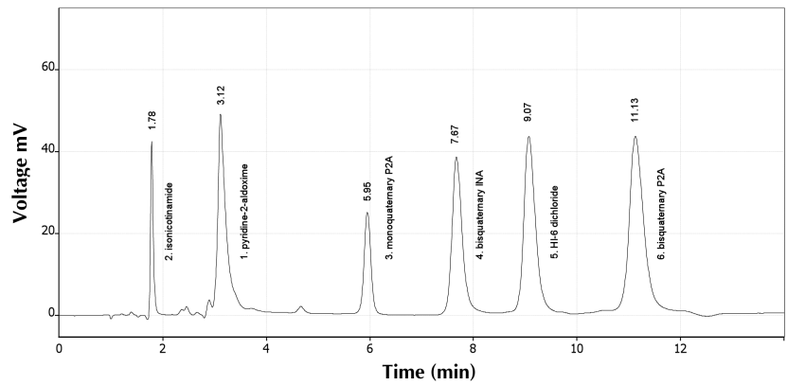

Figure 2. Column: Lichrospher 60 RP-select B (250 × 4 mm, 5 µm). Mobile phase: 24% acetonitrile–76% water (v/v) containing 8 mM of octane-1-sulphonic acid sodium salt and 2 mM of tetramethylammonium chloride. Flow rate: 1.5 mL/min. Detection: 277 nm.

Table II. HPLC Properties of Tested Compounds

Compound No.	Retention time (min) RP select B	Equation of the calibration curve	Correlation coefficient	Standard deviation	Retention time (min) Spherisorb CN
1	3.12	$y = 3.44420x$	$r = 0.999903$	2.167	3.72
2	1.78	$y = 1.25444x$	$r = 0.999612$	1.389	3.10
3	5.95	not measured	–	–	5.31
4	11.13	$y = 6.09266x$	$r = 0.999741$	5.501	4.01
5	7.67	$y = 3.97372x$	$r = 0.999688$	3.848	3.19
6	9.07	$y = 5.10408x$	$r = 0.999996$	0.523	3.57
7	9.01	$y = 5.09074x$	$r = 0.999912$	0.590	3.56

Experimental

Chemicals

Acetonitrile gradient-grade LiChrosolv, octane-1-sulphonic acid sodium salt (99%), sodium acetate (99%), and glacial acetic acid (100%) were purchased from Merck (Darmstadt, Germany). Tetramethylammonium chloride (97%), pyridine-2-aldoxime (99%), and isonicotinamide (99%) were purchased from Aldrich (Steinheim, Germany). Water was reverse osmosis pure. HI-6 and its intermediates were synthetized previously in our laboratory (13–14,18–19). Their characteristics are given in Table I. Monoquaternary pyridine-2-aldoxime was not pure because of its very low stability. Its purification is not possible for this reason.

Apparatus

The HPLC system consisted of a P200 gradient pump (Spectra-Physics Analytical, Fremont, CA), a 10-µL loop 7125 injection valve (Rheodyne, Cotati, Rohnert Park, CA), a UV 1000 detector (Spectra-Physics Analytical), and a CSW Chromatography Station 1.5 software (DataApex, Praha, Czech Republic).

Sample preparations

Calibration curves for all compounds except of unstable monoquaternary pyridine-2-aldoxime were measured. 500 µM of each compound in 24% water–acetonitrile was prepared and diluted to 250, 100, and 10 µM. Each concentration was measured three times.

Chromatographic conditions

For analyses and calibration, a Lichrospher 60 RP-select B column (250 × 4 mm i.d., 5 µm) (Merck, Darmstadt, Germany) was used. The mobile phase was 24% acetonitrile and 76% water (v/v), containing 8 mM octane-1-sulphonic acid sodium salt and 2 mM tetramethylammonium chloride. It was delivered isocratically at a flow rate of 1.5 mL/min. The absorbance was measured at a compromise wavelength 277 nm. All chromatograms were obtained at room temperature (25°C) (Figure 2) (20–22). All calibration curves are defined by response base – area, compound type – ordinary, curve fit type – linear, origin – curve passes through the origin.

For comparison, a Waters Spherisorb Cyano column (250 × 4.6 mm i.d., 5 µm) (Supelco Inc., Bellefonte, PA) without the effect of ion-pairing reagent in mobile phase was used. The mobile phase was phosphate buffer (pH 2) containing

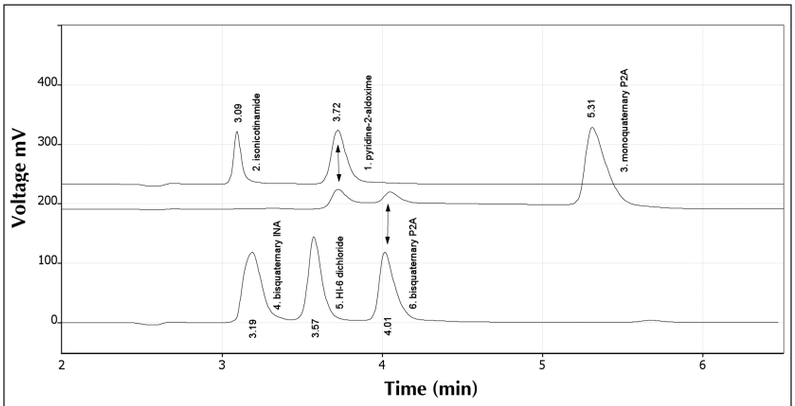

Figure 3. Column: Waters Spherisorb Cyano (250 × 4.6 mm, 5 µm). Mobile phase: phosphate buffer (25 mM K_2HPO_4 and 30 mM H_3PO_4) pH 2, containing 5% of acetonitrile (v/v). Flow rate: 1 mL/min. Detection: 277 nm.

5% of acetonitrile (v/v). The buffer was prepared with 25 mM K_2HPO_4 and 30 mM H_3PO_4. It was delivered isocratically at a flow rate of 1 mL/min. The absorbance was measured at a compromise wavelength 277 nm. All chromatograms were obtained at room temperature (25°C) (Figure 3).

Results and Discussion

In this article, the development of a quick and simple HPLC method with UV detection for identification of compounds considered as parent compounds, intermediates, and by-products of HI-6 synthesis was described. Our method could follow every reaction step within the synthesis of acetylcholinesterase reactivator HI-6, which is among the best antidotes against nerve agents. All compounds that could arise within HI-6 synthesis were very well-separated (Table II). They could be detected until the measured detection limit 1.0 µM. The detectable amount was 0.5 µM of a compound in a sample. The whole analysis took just 13 min. Calibration curve for monoquaternary intermediate was not measured because of its low stability. This compound fully decomposed in this mobile phase within 30 min. However, its response is comparable with those of its neighbor-tested compounds.

In our study, we have developed a method that does not use ion-pairing reagents approach. The basicity of the oxime nitrogen causing strong tailing of the peaks was annulated by the decrease of pH to 2. However, at this pH, retention times of parent substances and products are similar and thus overlap in the chromatogram (Figure 3). This method is better for monitoring of single substances in tissues and body fluids within the pharmacokinetic experimen because it is quick and cheap.

Conclusions

In conclusion, we have developed a simple method for identification of all compounds arising within the synthesis of acetylcholinesterase reactivator HI-6. Because of the recent threat of chemical warfare agent misuse, such method could properly help scientists and developers who are aimed in production of this antidote throughout the world.

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