International Journal of Analytical Mass Spectrometry and Chromatography, 2023, 11, 1-10
https://www.scirp.org/journal/ijamsc
ISSN Online: 2332-1776
ISSN Print: 2332-1768

Review: Practical Use of a Neurophysiological Detector and the Protocol for High-Performance Liquid Chromatography

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How to cite this paper: Brondz, I. (2023) Review: Practical Use of a Neurophysiological Detector and the Protocol for High-Performance Liquid Chromatography. *International Journal of Analytical Mass Spectrometry and Chromatography*, **11**, 1-10.
https://doi.org/10.4236/ijamsc.2023.111001

Received: February 1, 2023
Accepted: March 28, 2023
Published: March 31, 2023

Abstract

The main aim for discovery and development of the neurophysiological detector was detection of the production' seats and criminal use of poisons in warfare. Phosphor-organic (PO) substances with acetylcholinesterase-blocking effects are prohibited in warfare by international law (Geneva Protocol. https://www.un.org/disarmament/wmd/bio/1925-geneva-protocol/). Monitoring PO analogs with acetylcholinesterase-blocking effects and their degradation products in water and soil can provide clues to unlawful production sites and the possible use of POs in warfare. Attempts to analyze POs by derivatization have had a low ability to detect them. A neurophysiological detector (NPD)-high-performance liquid chromatography (HPLC) system was developed for specific detection with high detection ability. The first official presentation of our NPD was at the 3rd *International Symposium on Separation in BioSciences SBS* 2003: *A* 100 *Years of Chromatography*, May 13-18, 2003, in Moscow, Russia. The NPD in connection to HPLC was developed 14 years before the presentation at the SBS in 2003. Initially, NPD combined with an HPLC system was developed for intelligence services and only for use in monitoring and espionage against the unlawful production of neuroparalytic agents, as explained in this article. NPD combined with an HPLC system was developed in Umeå, Sweden, in 1987-89; the protocol was further developed in Statens Plantevern Institutt, Ås, Norway, in 1990-92. NPD may have great utility during the current period of active warfare in Europe. The initial challenge was detecting unlawful production and use of PO compounds and their metabolites that can potentially block acetylcholinesterase. The sensor in NPD can detect and monitor substances such as tabun, soman, and modern PO poisons used in military applications. This article describes the history of the development of NPD and its aim as a sensitive sensor in detecting PO substances with acetylcholinesterase-blocking effects.

Keywords

Neurophysiological Detector, Phosphor-Organic Substances, Tabun, Soman, Criminal Use of Poisons in Warfare

1. Introduction

The initial task was to develop a protocol, analyses, and equipment to detect highly toxic PO substances and their metabolites in water and soil. The aim was to detect the minimal possible concentration of a PO substance that may be used in warfare. The use of a non-enriching method in an aqueous phase was preferable. Chromatography and electrophoresis are two main approaches to separating individual substances within complex natural or artificial mixtures. High-performance liquid chromatography (HPLC) is the best option for separating PO target substances from wastewater. Here, we will discuss the chromatographic approach. The target substance should be detected and recognized unequivocally. Another option is to use gas chromatography (GC). Due to the way GC capillary columns are constructed, they provide a high concentration of the target substance (TS) in the separated chromatographic peak. The injection volume ranged from 0.1 to 2 μL. The concentration of the TS in the injection volume can be low. The nature of the detector plays a vital role in detecting the minimal amount of TS. Mass spectrometry (MS) detection is preferable, especially for detecting a single ion. MS provides high sensitivity and assurance of the nature of TS. However, using GC appears restrictive because of the requirements for high temperature, volatility, and some other parameters. These restrictions can be overcome by using supercritical fluid chromatography (SFC) equipped with multiple detectors, including MS-ultraviolet and corona-charged aerosol detection (CCAD) [1] [2] [3], or by using GC-MS with supersonic molecular beams (SMB) [4]. However, using water injection in a GC equipped with a capillary column is not feasible. In these cases, using HPLC, especially combined with MS as a detector, is a feasible solution [5]. Here, the use of HPLC was necessary because the TSs were in the aqueous phase. Extraction in the organic phase is possible; however, the loss and dilution of TSs during extraction require enrichment. During the extraction and concentration, losses of TS are inadmissible. We chose diazinon as a model substance for studying MDC. Diazinon is an agricultural representative of PO substances with an acetylcholinesterase-blocking effect [6].

2. The History of Chemical Warfare and the Prohibition of Poisonous Ammunition

The history of chemical warfare is described in: A Brief History of Chemical and Biological Weapons.
https://web.archive.org/web/20041205051646/http://www.cbwinfo.com/History/History.html

The use of chemical warfare was known to samurais in Japan and described in ancient manuscripts by Greeks. Arsenical smoke was described in China's weapon arsenal as early as 1000 BC [7]. At the First Hague Conference on August 24, 1898, Russian Tsar Nicholas II and the foreign minister of Russia, Count Mikhail Nikolayevich Muravyov, initiated the international Hague Conventions in 1899. The Hague Conventions of 1899 and 1907 were based on three main treaties and three additional declarations:

1) Convention for the Pacific Settlement of International Disputes;

2) Convention with respect to the Laws and Customs of War on Land (which was based on the Geneva Convention of 1864);

3) Convention for the Adaptation to Maritime Warfare of the Principles of the Geneva Convention of August 22, 1864;

4) Declaration concerning the Prohibition of the Discharge of Projectiles and Explosives from Balloons or by Other New Analogous Methods;

5) Declaration concerning the Prohibition of the Use of Projectiles with the Sole Object to Spread Asphyxiating Poisonous Gases; and

6) Declaration concerning the Prohibition of the Use of Bullets which can Easily Expand or Change their Form inside the Human Body such as Bullets with a Hard Covering which does not Completely Cover the Core or containing Indentations.

Russian Tsar Nicholas II initiated this conference and supported the convention because Russia was the first European country whose troops experienced the effects of chemical warfare. This was during the Crimean War at the siege of Sevastopol. Tsar Nicholas II predicted the looming great war and wanted to avoid unnecessary evil for his troops. The prohibition to use the projectiles "the sole object of which is the diffusion of asphyxiating or deleterious gases" was declared by The Hague Convention of 1907.
https://www.loc.gov/rr/frd/Military_Law/pdf/Hague-Peace-Conference_1899.pdf.
During the First World War (WWI), France was the first to use chemical weapons, such as the tear gases ethyl bromoacetate and chloroacetone. More than one million combatants died during WWI because of the use of poisonous chemicals. Both sides of the conflict used poisonous chemicals such as chlorine, phosgene, mustard gas, lewisite (β-chlorovinyldichloroarsine), adamsite (diphenylaminechloroarsine), Clark I (diphenylchloroarsine), and Clark II (diphenylcyanoarsine). These horrible events initiated an international outcry for the regulation or prevention of chemical and bacteriological warfare. On June 17, 1925, the so-called Geneva Protocol was signed as a Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Poisonous Gases, and of Bacteriological Methods of Warfare, or so-called Chemical Weapons Convention—OPCW,
https://www.opcw.org/sites/default/files/documents/CWC/CWC_en.pdf. During the following years, up to the beginning of World War II (WWII), several efforts were made to prohibit the use of chemical and biological weapons, not only in theaters of war against troops, but also against civilians where they have demon-

strated against governments. Some consensus was reached to prohibit the use of chemical and biological weapons against troops on battlefields. France protested and prevented the assertion of prohibiting the use of chemical weapons against civilians, especially in civil riots [8]. One of the reasons why Nazis were not accused at the Nuremberg Trials for using poisonous gases and biological agents against partisans in Odesa, Kerch, and Sevastopol, and against other civilians participating in riots, was French opposition to prohibit the use of chemical weapons against civilians (see ref. [8]). The second reason was the resistance of the USA to draft a proposal opposing the introduction of the Colorado potato beetle (*Leptinotarsa decemlineata*) onto the soil of Axis powers as biological warfare. The third reason was Stalin's position to defend the countries that came under the control of the Soviets (Yalta Conference agreement). Stalin's position prevented drafting accusations of the use of poisonous ammunition by the Polish army against German troops in 1939 [9]. Stalin and the USSR delivered a long list of questions that must not be asked at the Nuremberg trial. This information [9] is based on publications in the following sources: The International Military Tribunal for Germany; Contents of The Nuremberg Trials Collection, http://avalon.law.yale.edu/subject_menus/imt.asp; and The Allies and the Use of Gas in WWII http://rense.com/general83/gas.htm [10]. The incident is described in [10] as follows: *"The first incident involving poisonous gas in WWII occurred on the evening of Friday, September* 8, 1939, *in the village of Jaslo in the south of Poland*. (5) *Polish troops had tried to blow up a railway bridge over the river Jasiolka. The Poles had used a chemical bomb* (6). *As German soldiers from* 1. *Gebirges–Pionere Battalion* 82 (*a battalion of engineer infantry*) *came to clear the bridge it exploded. The engineer soldiers found the Poles had used a chemical explosive—but that explosive had not exploded—as it exploded*, 14 *soldiers became mustard gas victims, two of the soldiers died*." Accusations against Nazi in the use of poisonous munitions were dropped. The most heavily weighted argument was that Nazis did not use poisonous munitions against troops on the battlefields [11]. The Nazis did not use poisonous munitions on the Normandy beachhead; the use of nerve gas by Nazis could have seriously impeded the Allies and possibly caused the invasion to fail altogether. The absence of accusations for the use of bacteriological weapons by the Red Army should also be mentioned. The Red Army used bacteriological weapons against the Nazis during the Stalingrad offensive by Paulus's army. The Red Army spread rats with tularemia in fields in front of German forces. Numerous cases of so-called "hay's disease" were detected among the German troops. This epidemic caused the approximately two-week hold on the Stalingrad offensive by Paulus's army. The absence of any accusation was because the Soviet delegation delivered a long list of questions that must not be discussed at the Nuremberg Trials. The cases mentioned above show that the global community relaxed accusations concerning one side of these crimes and sensitivity concerning the other side. However, monitoring production and chemical weapon use is essential because not all countries accepted The Geneva Protocol [12], even long after 1925. The USA

used chemicals in warfare during the Korean and Vietnam wars. The Geneva Protocol was ratified by the USA only in 1990. Despite ratification, many countries, such as Japan, violated The Protocol during WWII, and later Iraq during the Iraq-Iranian war.

3. Sites of Suspicion and Attention

After the end of the WWI and the capitulation of Germany by the Treaty of Versailles, and The Geneva Protocol in 1925, many countries among the members who signed the protocol and many who did not sign it developed centers of chemical and biological warfare research, such as the Chemical Biological Centre (KBC) in Umeå, Sweden; the Defence Chemical, Biological, Radiological and Nuclear Centre (Defence CBRN Center), at Winterbourne Gunner, UK; Shikhany-2, Saratov Oblast, Russia, and many others. The Defence CBRN Centre site was established as an element of the Porton Down research facility in 1917. The military chemical base at "Shikhany-2" was established as early as 1924 as a center of Soviet chemical warfare activities. It became a part of the secret Tomka project (under codename Vol'sk-18). The Tomka project was a Soviet-German joint chemical warfare experimental center and laboratories. Among the countries that signed The Geneva Protocol in 1925 was the Weimar Republic. The Weimar Republic—Germany—was forbidden from undertaking tests with chemical warfare agents or developing associated delivery systems by the Treaty of Versailles. The Tomka project was operated from 1926 to 1933 to circumvent the Treaty of Versailles and The Geneva Protocol. After Hitler came to power in Germany, the Tomka project was closed. However, the center in Shikhany-2 has functioned up to the present. It was concerning that Shikhany-2 is a source of the Novichok agent. Professor Kurt Andeson said that the water sample for analysis presented to the author was from the region of Vol'sk, a small settlement close to Shikhany, and should be analyzed for the presence of POs and their degradation products.

4. The Projects in Telemark Central Hospital, Norway, and at Umeå University, Sweden

During their doctoral study (the PhD project entitled "The development of the chemotaxonomy for microorganisms," conducted at the University of Oslo, Norway), the author also worked at the Telemark Central Hospital, Porsgrunn, Norway, with another project, "Measurements of pesticide aerosol exposure of workers in industry and agriculture."

The Problems with Aerosol Monitoring and Introduction of Rubidium as a Tracer

In the Telemark Central Hospital, Norway, the author was responsible for analyzing the exposure of agricultural workers to pesticides. The author was required to analyze several thousand doses of exposure to aerosols from the tractors that spread pesticides on fields and in greenhouses for the PO insecticides

dispersed as foam, aerosols, dust, and smoke. To measure the use of POs, aerosols were collected on static objects with known surface areas, or the liquid phase was collected inside impinges. There are several problems with this methodology. There is a lack of persistence of distinct POs in the environment. Diazinon, malathion, and many others are easily oxidized in humid air, especially under irradiation by sunlight. Diazinon and malathion become diazoxon and malaoxon, respectively (**Figure 1**). This transformation occurs because the sulfur connected to the phosphorous is easily oxidized and exchanged with oxygen. After a short time, exposure of these substances to sunlight and humidity gave two different peaks with different retention times (R_t), and the degradation molecules gave a different response in the detector by using HPLC or GC. Quantification is difficult, and analyses require considerable time. For a single HPLC analysis, the time was up to 25 min, and for GC, up to 7 min. In the study, it was necessary to analyze several thousands of samples. The dose of exposure to pesticide could be calculated from the amount of liquid dispersed as aerosol because the concentration of pesticide per milliliter of aerosol was known. By adding liquid together with pesticide to a known amount of stable spore metal Rb that was easy to analyze, a short analysis time to calculate the dose of exposure was possible. The author used rubidium salt, which is absent in the environment, and gave a strong response in atomic absorption spectrometry (AAS) [13]. The time needed for AAS analysis was less than 1 min.

5. The Purposes of the NPD–HPLC System

After fulfilling PhD requirements, the author was invited to Umeå University, Sweden to work with Docent Gjøran Blomquist on a project of fuser development of the chemotaxonomy for microorganisms (fungi) [14] [15] and with Prof. Kurt Anderson and Prof. Kolmodin-Hedman on a project to study exposure and analyses of PO insecticides [6]. The project duration was from 1987 to 1990. Prof. Kurt Anderson proposed participation in his project to analyze military PO substances and their degradation products, which he has from the Chemical Biological Centre (KBC) in Umeå
https://www.umu.se/en/chemical-biological-centre/, e-mail: info.kbc@umu.se.

The German Federal Intelligence Service (BND) obtained a sample of the Novichok agent (military PO), and the sample was transferred to Sweden for analysis. The Novichok agent belongs to the class of organophosphate acetylcholinesterase inhibitors. The sensitivity to the TS obtained in analyses described in [6] for insecticides and especially for their degradation products was not satisfactory for military POs. Because POs act on an acetylcholinesterase as an inhibitor of the enzyme in neurons, the author proposed to measure the reactions of the neurons directly. This project required the analysis of extremely small concentrations of POs in the aqueous phase without dilution or derivatization. From these restrictions came the idea of using HPLC with neurons as detectors. The architecture of the instrumental construction is presented in **Figure 2**. The

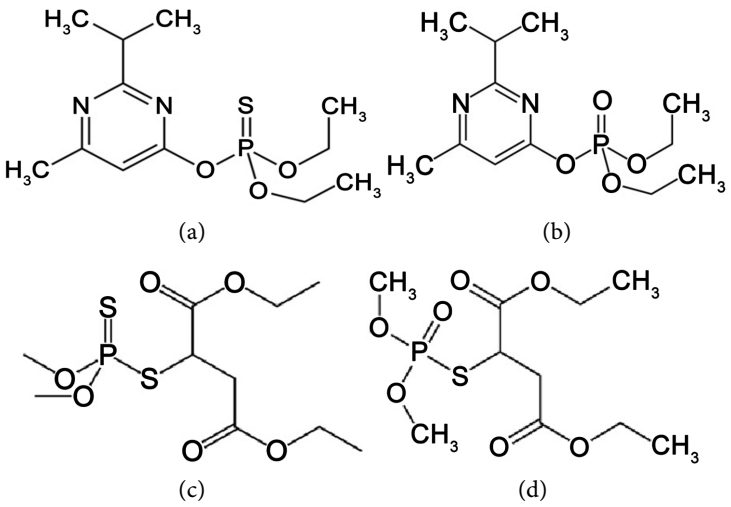

Figure 1. Structures of POs: (a) Diazinon; (b) Diazoxon; (c) Malathion; (d) Malaoxon.

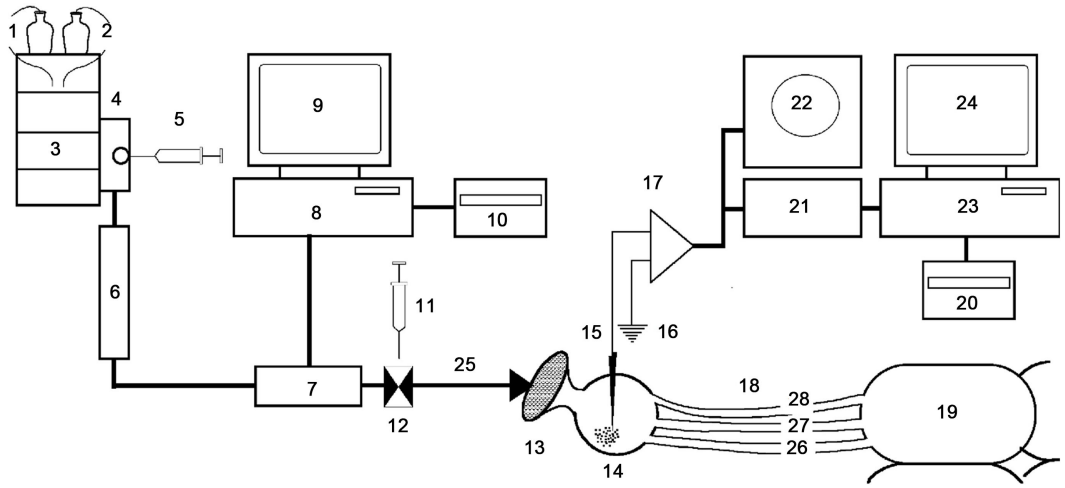

Figure 2. A schematic drawing of the experimental set-up of the HPLC equipped with DAD and NPD. The set-up included (1) reagent bottle 1 with APW; (2) reagent bottle 2 APW with 10 ppm of diazinon; (3) the HPLC system; (4) a Rheodyne injector; (5) a syringe with a mixture of sex hormones or a syringe with fish skin extract; (6) column; (7) the DAD; (8) the processor for HPLC; (9) a PC screen for the HPLC system; (10) data acquisition printer 1 for the HPLC system; (11) a syringe with a sample for analyses; (12) a microvalve (13) an olfactory epithelium (OE); (14) an olfactory bulb (OB); (15) a tungsten (wolf-ram) electrode; (16) an earth ground connection; (17) an amplifier; (18) olfactory tracts 26, 27, 28); (19) telencephalon; (20) data acquisition printer 2 for the NPD system; (21) the AD converter; (22) an oscilloscope; (23) the processor for the NPD system; (24) a monitor for the NPD system; (25) transference tubing.

influence of POs on humans and on other living organisms is generally described in [9]; the principal procedure is described in [16] and [17] as the preparation of biological material and the preparation of chemicals that were used in the analyses. The evolution of the system and the consequences of exposure of humans to POs are described in [16]. The monitoring of warfare analogs and their degradation products in water and soil can give (and gave (unpublished results)) a clue of production sites and possible use of chemical OPs in warfare.

The NPD–HPLC system was developed to monitor the use of POs in warfare and PO production sites, the exposure of agricultural workers to agricultural insecticides, and the exposure of industrial workers and the environment to POs. The classical measurement means that one of several compounds delivered as foam, aerosol, dust, and smoke are analyzed to determine the concentration. The exposures were calculated by time and areal exposure. The calculation of exposures was through contact with previously exposed equipment or things, by contamination of water and soil (e.g., water filtration through soil).

6. Taking the Samples

The correctness of the results of the analyses depends on the first step, which is a correct sample-taking procedure. Most pesticides, xenobiotics, and military warfare agents are not exclusive. All these substances can appear in the water of creeks, rivers, and lakes. The place of contamination and progression to the sample-taking point can be determined by using a tracer (in our case, rubidium salt). Samples in the project were evaluated in Norway by studying the movement of pesticides by water transport through soil [18] [19] [20]. The studies were of *Pesticide runoff from agricultural land*, *Vertical transport of pesticide in soil in field experiments*, and *Mobility and degradation of pesticides in column experiments*.

7. Conclusion

The system using NPD combined with HPLC is not designed for a long series of analyses. The use of NPD has several restrictions that impose limits on its use: the sensor is part of a living creature-fish, and is not an industrial product. Therefore, preparing the sensor demands a skilled and well-trained veterinary surgeon. The sensor is a short-lived device, and the HPLC columns adopted should be for the aqueous phase. The NPD has several advantages: in contrast with bulk- and solute-property detectors, the NPD is selective and sensitive to distinct chemical molecules that have a specific physiological action in living organisms, such as pheromones, neurotoxins, neurotransmitters, and narcotics. NPD has a very high sensitivity of 1.4×10^{-15} mmole/mL diazoxon. Its low sensitivity toward other substances permits good distinguishing of the target substances from the background of other substances usually present in the aqueous phase and, therefore, in water extracts of soil, in urine, and in blood. Despite its lack of industrial production and other restrictions, NPD is a useful tool for detecting unlawful production and use of toxic warfare agents in military settings, some narcotics in forensic medicine, and doping in sports.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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