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Review

Review

A Brief Overview of HPLC–MS Analysis of Alkyl Methylphosphonic Acid Degradation Products of Nerve Agents

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Abstract

The analysis of degradation products from the classic chemical warfare nerve agents by high-performance liquid chromatography–mass spectrometry has been of much interest in recent years owing to the possible use as a terrorist weapon, and the incidents of chemical weapon usage in recent years in war torn countries. The alkyl methylphosphonic acid degradation products are of a particular interest, and they represent a specific chromatographic technical challenge for use in typical separation systems. Various published methods are summarized in this review and some of the problems associated with the analysis of these compounds are discussed. Future trends of the analysis in this area of research are also considered.

Introduction and Background

The need for improved methodology for the detection and identification of the presence of chemical warfare agents (CWAs) has become more relevant in recent years due to increased public awareness and the concern about their use. Increases in terrorist activity around the world in the last few decades, as well as the possible use by some countries not abiding by the various international conventions on the use of chemical weapons (1), has increased the demand for rapid and sensitive methods for the analysis of both the active agents and their degradation products. The nerve agent sarin was used in the 1995 Tokyo subway terrorist attack by a cult resulted in the death of 12 people and the injury or exposure of ~5,000 (2, 3). Nerve agents were also used in 2013 during hostilities in Damascus, Syria causing many civilian casualties and had lasting post attack health effects (4–6). The interest in improving analytical methods has been further motivated by the Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction (1). Although this convention came into force in 1997, some countries in the Mideast and North Korea (7) are believed to use or stockpile CWAs.

The detection of CWAs and their related degradation products have utilized a broad range of analytical techniques and have been reviewed extensively in the literature (8–10). These techniques have included, for example, gas chromatography (GC), high-performance liquid chromatography (HPLC), mass spectrometry (MS), chemical determination, sensors and immunoassays. However, a more detailed and focused perspective, overviewed herein, on the alkyl methyl phosphonic acid degradation products of nerve agents determined by means of HPLC–MS is necessary. The methodologies for CWAs and their degradation products have several main goals. These include the identification of the agent to ensure appropriate medical treatment of the exposed, the monitoring of first responders or cleanup workers operating in areas where chemical weapons have be used, and the investigation of alleged use of chemical weapons for forensic conformation. These analysis can consist of environmental testing or biomonitoring.

The nerve agents represent the class of the most toxic CWAs and are among the most potent toxicants known. Munro *et al.* (11) covered the sources, the environmental fate and the biological toxicity of these and other CWAs in an extensive review and will be only briefly summarized below. Chemically, the nerve agents are alkyl

phosphonic acid esters that elicit their toxic effect by the irreversible inhibition of the enzyme cholinesterase. This results in the accumulation of acetylcholine and, in turn, a continuous stimulation of the nervous system occurs. Vital autonomic functions eventually fail after a lethal exposure level to a nerve agent. The nerve agents of interest here, are divided into two classes, the G agents and the V agents (Table I and Figure 1).

Historically, the G agents were first synthesized in Germany just prior to the start of World War II by chemist Gerhard Schrader (12, 13). The G agents produce alkyl methylphosphonic acid degradation products. These G agents include sarin (GB, isopropyl methylphosphonofluoridate), soman (GD, pinacolyl methylphosphonofluoridate) and cyclohexyl sarin (GF, cyclohexyl methylphosphonofluoridate). Tabun is another G agent, but it is structurally different and does not hydrolyze to form a methylphosphonic acid analog, and thus, will not be discussed here. The V agents were developed during the post-World War II period, first in Great Britain (13). While employed at Imperial Chemical Industries, Ranajit Ghosh and J.F. Newman discovered the pesticide Amiton, *S*-[2-(diethylamino)ethyl] *O*,*O*-diethyl phosphonothioate (VG, Figure 1). Other V agents were being studied as weapons by Great Britain such as VE (*S*-(diethylamino)ethyl *O*-ethyl ethyl phosphonothioate) and VM (*S*-(diethylamino)ethyl *O*-ethyl methyl phosphonothioate), but VX (*O*-ethyl-*S*-[2-(diisopropylamino) ethyl] methyl phosphonothioate) was found to be substantially more lethal and eventually fully weaponized (12–14). Russia eventually produced their own version of VX known as Russian VX (RVX, *O*-isobutyl-*S*-[2-(diethylamino) ethyl] methyl phosphonothioate) as well as the Chinese developing Chinese VX (CVX, O-butyl-S-[2-(diethylamino)ethyl] methyl phosphonothioate). The two V agents, VX and Russian VX, are the focus of most of the analytical work in the literature, and both are among the most lethal substances ever produced by man. According to Munro *et al.*'s review (11), the nerve agents tend to have a low persistence in the general environment as compared to other CWAs (i.e. the nitrogen mustards). The G agents are volatile and present a vapor hazard, but in turn, dissipate more quickly in the open environment, and they are more susceptible to hydrolysis than the V agents. The V agents also are the most persistent nerve agents on surfaces and they have the slowest hydrolysis rate in surface water. All the nerve agents can act by dermal, oral or inhalation routes of exposure. The mechanism for the anticholinesterase action of these organophosphonic compounds is based upon the phosphorylation of the enzyme's active site. The phosphorylated enzyme is highly stable, and thus, the cholinesterase is irreversibly inhibited (15).

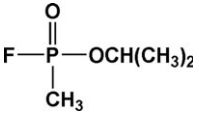
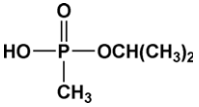
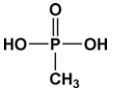
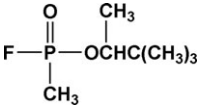
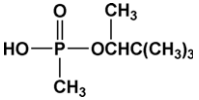
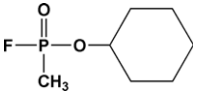
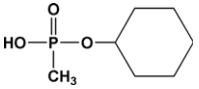
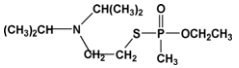
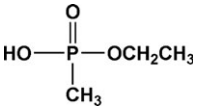
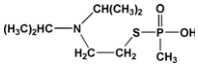
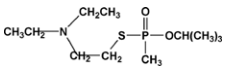
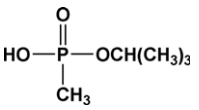
Each nerve agent has an initial unique degradation product (Table II). Isopropyl methylphosphonic acid (iPMPA) is an intermediate degradation product of Sarin, pinacolyl methylphosphonic acid (PinMPA) is an intermediate degradation product of Soman and cyclohexyl methylphosphonic acid (CHMPA) is the corresponding intermediate product of cyclohexyl sarin. VX has the main signature degradation product of ethyl methylphosphonic acid (EMPA) generally at pH levels of <6 and >10 (11). VX can degrade to EA2192 (*S*-[2-(diispropylamino)ethyl] hydrogen methylphosphonothioate, see Table I), which unlike many of the other nerve agent degradation products, has a toxicity and biological activity similar to the parent VX. Russian VX initially degrades to isobutyl methylphosphonic acid (iBMPA). The final common degradation product of sarin, soman, cyclohexyl sarin, VX and Russian VX is methylphosphonic acid (MPA), and the degradation routes of the compounds have been discussed extensively in the literature (3, 11, 16–18). Most of the simple alkyl methylphosphonic acids are not cholinesterase inhibitors and are of low toxicity.

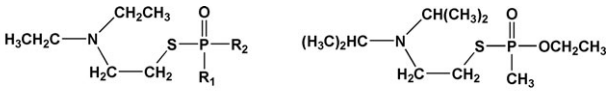
Much of the analytical interest is in the detection and quantification of the alkyl methylphosphonic acids, which are a product of degradation or metabolic action from the classic nerve agents. This has led to the analytical need to detect these compounds in soil, surface waters, contaminated surfaces and biological specimens. Additionally, biomonitoring of exposure to CWAs is of great importance (9, 19). Analysis of the alkyl methylphosphonic acid degradation products is problematic in that they are polar and are without strong chromophores or fluorophores. These factors result in some limitations, or at least considerations, for the general use of high-performance liquid chromatographic systems with mass spectrometric detection which will be discussed. Other reviews have covered HPLC analysis in the past (2, 3, 16, 20, 21); however, a focused perspective is covered here in with the alkyl methylphophonic acids and HPLC–MS. HPLC analysis utilizing MS detection has generally been found to be suitable for the analysis of both biological and environmental samples, following appropriate sample preparation without the need for chemical derivatization. Some methods described in the literature have approached the analysis of these alkyl methylphosphonic acids by "dilute-and-shoot" procedures (22), although there are additional challenges of contending with the general complexity of sample matrices encountered. This review will summarize some of the applications of HPLC–MS analysis of the alkyl methylphosphonic compounds in the literature.

Mass Spectrometric Detection

The basic function of the mass spectrometer is to measure the mass-to-charge ratios of analyte ions, and the various design configurations of mass spectrometers have been described in detail elsewhere (23, 24). Various forms of MS have been used for the detection of the alkyl methylphosphonic acids that are reported in the literature. MS has many advantages for use in detecting and measuring these compounds; MS possesses high sensitivity and adds a higher degree of specificity, the ability of the technique to accurately measure the target analyte while minimizing the possibility of interferences. This advantage of MS usually eliminates the need for a derivatization step to the overall analysis procedure. This factor leads to more timely results and most probably a lower cost for avoidance of sample preparation for the chemical derivatization step. Tandem mass spectrometry (MS–MS) gives the best performance with respect to both sensitivity and specificity. The alkyl methylphosphonic acids lack useable UV absorbance chromophores and do not fluoresce, thus, MS detection offers the best alternative. However, it must be kept in mind that overall method sensitivity is based on both the detector as well as sample pre-concentration steps. Furthermore, in the case of environmental or biological samples, the sample matrix is complex and the specificity of detection offered by MS becomes a significant advantage. Although there have been studies reporting the use of inductively coupled plasma-MS (ICP-MS) detection for alkyl methylphosphonic acid compounds (25), the more common forms of ionization for MS sample introduction, either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), are typically used. Black *et al.*'s research (17, 18, 26) represents some of the more extensive early work in method development to detect and quantify the alkyl methylphosphonic acids and these studies require acknowledgment in any review. Furthermore, Table II of this review summarizes the research of others pertaining to the analysis of the alkyl methylphosphonic acids from nerve agent degradation.

Table I. Structures of the Nerve Agents and Their Key Methylphosphonic Acid Degradation Products

CW common name/ abbreviation	CW compound	CW structure	Major degradation product(s)	Degradation product abbreviation	Degradation product structure
G Agents					
Sarin/GB	Isopropyl methyl phosphono-fluoridate		isopropyl methylphosphonic acid	iPMPA	
			methylphosphonic acid	MPA	
Soman/GD					
	Pinacolyl methyl phosphono-fluroridate		pinacolyl methylphosphonic acid	PinMPA	
			methylphosphonic acid	MPA	See above
Cyclohexylsarin/GF	Cyclohexyl methyl phosphono-fluoridate		cyclohexyl methylphosphonic acid	CHMPA	
			methylphosphonic acid	MPA	See above
V Agents					
VX	*O*-ethyl-*S*-[2-(diisopropylamino)ethyl] methylphosphonothioate		ethyl methylphosphonic acid	EMPA	
			S-[2-(diispropyl-amino)ethyl] hydrogen methylphosphono-thioate	EA 2,192	
			methylphosphonic acid	MPA	See above
Russian VX or RVX	*O*-isobutyl-*S*-[2-(diethylamino)ethyl] methylphosphonothioate		isobutyl methylphosphonic acid	iBMPA	
			methylphosphonic acid	MPA	See above



VE: $R_1 = CH_2CH_3$ $R_2 = OCH_2CH_3$

VG: $R_1 = OCH_2CH_3$ $R_2 = OCH_2CH_3$

VM: $R_1 = CH_3$ $R_2 = OCH_2CH_3$

CVX: $R_1 = CH_3$ $R_2 = OCH_2CH_2CH_2CH_3$

RVX: $R_1 = CH_2CH_3$ $R_2 = OCH(CH_3)_3$

VX

Figure 1. Structures of the V agents.

The two most common ion sources for HPLC–MS determination and measurement of the alkyl methylphosphonic acids are ESI and APCI. ESI and APCI are both "soft" ionization techniques and each have specific advantages. The ESI technique has become the ion source of choice for most general HPLC–MS work over the older interfaces such as thermospray. In ESI, the effluent from the HPLC column is passed through a small capillary or jet held at a high electrical potential. This results in electrostatic nebulization of the liquid. During desolvation of the product droplets, the electric field on the droplet surface leads to ejection of charged analyte ions upon final evaporation. In APCI, the effluent from the HPLC column is heated and sprayed with a high flow of nitrogen from a nebulizer that generates an aerosol. This aerosol is subjected to a corona discharge to form ions of the analytes. In APCI, the ionization of the analytes takes place in gas phase, unlike ionization in ESI which takes place in the liquid phase. Finally, ICP, which is reported only once in the analysis of the alkyl methylphosphonic acids (25), is a "hard" ionization source. An ICP usually consists of a quartz torch constructed of concentric tubes surrounded by a copper load coil. The load coil is connected to a radio frequency generator that induces an oscillating magnetic field at the top of the torch. Plasma is formed while a spark is applied to the flowing argon gas stream to form gaseous ions. The free electrons created by this process are accelerated by the magnetic field and bombard other gas atoms, which in turn, cause further ionization to produce the plasma. This plasma operates at a temperature of roughly 8,000 K; analyte molecules are broken down to atomic ions for mass spectrometric detection.

These interfaces with HPLC make certain conditions necessary with respect to appropriate chromatographic mobile phases being employed. The ESI requires ionization of the analytes within the mobile phase; therefore, some aqueous component is generally required. Also, ESI and APCI can be susceptible to ion suppression or ion enhancement from sample matrices. The ICP is not compatible with mobile phases containing acetornitrile; therefore, and alternative organic modifier such as methanol would be required for a typical reversed-phase HPLC system.

General Chromatographic Challenges and Common Strategies

The main advantages of HPLC for use as a separation technique for the alkyl methylphosphonic acids are very clear. These degradation products are generally water-soluble and are polar. HPLC–MS can be used directly without a complicated derivatization procedure, thus making sample preparation much easier for an analysis method. Another added general benefit in avoiding chemical derivatization is that preliminary interpretation of fragmentation, in the case of unknowns, is much simpler with the underivatized analytes. Most methods cited in the literature employ reversed-phase HPLC, but there are studies having employed reversed-phase ion-pair as well as some cases of hydrophilic interaction liquid chromatography (HILIC) being used (See Table II).

The accurate determination of the alkyl methylphosphonic acid degradation products from environmental samples (surface water, soil, wipes from various surfaces, etc.) or biological samples (blood, blood serum, saliva, urine, etc.) creates interesting challenges for the analytical chemist. Although there is the common perception that the use of HPLC–MS, and specifically HPLC–MS/MS, can guarantee the lack of interferences and method specificity, in practice this may not be the case. Common problems of interferences and ion suppression from matrix effects can be encountered. Several strategies are traditionally employed in HPLC–MS design and development to counter matrix suppression problems. Stable isotope labeled internal standards (isotope dilution) such as deuterated or carbon-13 analogs of the alkyl methylphosphonic acids, can be used to counter matrix effects. The importance of initial sample cleanup and good chromatographic separation cannot be overstated. Removal of inorganic salts from a sample matrix can reduce ion suppression of an ESI source. Solid-phase extraction (SPE) or liquid-liquid extraction (LLE) can be used for sample cleanup of environmental or biological samples and reduce the possibility of ion suppression. Urine analysis can be particularly challenging owing to the number of acidic components in the matrix which can co-elute with the alkyl methylphosphonic acids and act as interferences. Mawhinney *et al.* (47) found a urine matrix related mass spectrum interference with EMPA; in an unpublished study, the National Institute for Occupational Safety and Health (NIOSH) found a urine matrix related mass spectrum interference with CHMPA. Roen *et al.* (34) actually employed an "online" SPE or pre-concentration switching system on the HPLC–MS/MS to load the sample matrix onto the HPLC column preceded by a salting-out assisted LLE procedure to prepare human serum and urine samples. The use of chromatographic gradients to increase the separation of sample analytes can reduce the probability of possible interferences although many of the works sited in the review did employ simple isocratic systems. Table II of this survey lists the chromatographic system, the sample matrix and the reference to the original work.

Reversed-phase HPLC

The majority of the work described in the chemical literature employs some type of reversed-phased system to separate the various alkyl methylphosphonic acids. Generally C18 columns or, more rarely, mixed C8–C18 columns have been employed. Mobile phase composition generally consists of either acetonitrile (ACN) or methanol (CH_3OH) as the organic modifier. Owing to the use of MS, volatile buffer or volatile acids are used in the mobile phases. These are generally either formic acid (HCOOH), acetic acid (CH_3COOH) or the ammonium buffer analogs ammonium formate ($HCOONH_4$) and ammonium acetate (CH_3COONH_4). Although trifluoroacetic acid (CF_3COOH) has been used in some researched separations, Black and Read (17) noted that a considerable improvement in sensitivity was obtained by using formic acid instead of trifluoroacetic acid in the mobile phase of a reversed-phase HPLC system when using ESI as the ionization source for the analysis of the alky methylphosphonic acids. Ion suppression from strong acids or from

Table II. HPLC–MS Analysis of Alkyl Methylphosphonic Acid Degradation Products of Nerve Agents

Type of chromatography	Column type and mobile phase composition	Ionization interface	Sample matrix	Target analytes	Reference
Reversed-phase	C8-C18/gradient/H_2O-ACN, CF_3COOH	APCI	Water and soil	MPA, iPMPA, PinMPA, CHMPA, EMPA, iBMPA, plus others	(26)
	C8-C18/gradient/H_2O-ACN, CF_3COOH	APCI and ESI	Water	MPA, iPMPA, PinMPA, CHMPA, EMPA, iBMPA, plus others	(17)
	C18/gradient/H_2O-CH_3OH, $HCOONH_4$	APCI	Water	MPA, iPMPA, PinMPA, CHMPA, EMPA, iBMPA, plus others	(18)
	C18/gradient/H_2O-CH_3OH, $HCOONH_4$	ESI	Water, soil	MPA, iPMPA, PinMPA, CHMPA, EMPA, iBMPA	(28)
	C18/isocratic/H_2O-ACN, HCOOH	APCI and ESI	Water	MPA	(29)
	C18/gradient/H_2O-CH_3OH, CH_3COOH	ESI	Saliva, urine	iPMPA, PinMPA, CHMPA, EMPA	(30)
	C18/gradient/H_2O-CH_3OH, HCOOH	ESI	Urine	iPMPA, PinMPA, CHMPA, EMPA, iBMPA	(31)
	C8-C18/gradient/H_2O-ACN, HCOOH	ESI	Pig blood serum	iPMPA, CHMPA	(32)
	C18/gradient/H_2O-ACN, HCOOH	ESI	Urine	iPMPA, PinMPA, EMPA, iBMPA	(22)
	C18/gradient/H_2O-CH_3OH, HCOOH	ESI	Nail clippings	iPMPA, PinMPA	(33)
	C18/gradient/H_2O-ACN, CH_3COONH_4	ESI	Soil	iPMPA, PinMPA, CHMPA, EMPA, iBMPA	(34)
	C18/gradient/H_2O-ACN, HCOOH	ESI	Blood Plasma	MPA	(35)
Reversed-phase ultra performance	C18/gradient/H_2O-ACN, HCOOH	ESI	Wiped Surfaces	MPA, iPMPA, PinMPA, EMPA	(36)
	C18/gradient/H_2O-CH_3OH, NH_4OH	ESI	Water	MPA, iPMPA, PinMPA, EMPA, plus others	(37)
Reversed-phase capillary column	C18/gradient/H_2O-ACN, CF_3COOH	ESI	Degraded VX reference sample	EMPA, EA 2,192, plus many others	(38)
	C18/gradient/H_2O-ACN, CF_3COOH	ESI	Soil Extracts	iPMPA, PinMPA	(39)
	C18/gradient/H_2O-ACN, CF_3COOH	ESI	Snow	MPA, iPMPA	(40)
Reversed-phase (with postcolumn derivatization)	C18/Isocratic/H_2O (100%), CH_3COONH_4	APCI	Reference samples	MPA	(41)
Reversed-phase ion-pair	C18/isocratic/H_2O-CH_3OH, various tetra alkyl ammonium salts with CH_3COONH_4	TS	Reference samples	MPA, iPMPA, PinMPA, CHMPA, EMPA	(27)
	C8/isocratic/H_2O-CH_3OH, various ion pair reagents with NH_4	ICP	Reference samples	MPA, iPMPA, EMPA	(25)
	C18/gradient/H2O-ACN, heptafluorobutric acid	ESI	Reference samples	MPA, iPMPA, EMPA and others	(16)
Ion exchange chromatography	Microbore-Anion Exchange/Isocratic/H_2O-ACN, HCOOH	ESI	Blood serum	iPMPA	(42)
	Anion Exchange /Isocratic/H_2O-ACN, various formic and acetic acid and buffers	ESI	Rat urine	MPA	(43)
Mixed mode	RP-anion exchange/isocratic/H_2O-ACN, $HCOONH_4$	ESI	Urine	MPA, iMPA	(44)
Adsorption	PGC/ isocratic and gradient/ various volatile acids	ESI	Tap water	MPA, iPMPA, PinMPA, CHMPA, EMPA, iBMPA and others	(45)
	PGC/gradient/MeOH-H_2O, $HCOONH_4$	ESI	Water	iPMPA, PinMPA, EMPA, iBMPA and others	(46)
HILIC	Isocratic/ACN-H_2O, $HCOONH_4$	ESI	Urine	iPMPA, PinMPA, CHMPA, EMPA, iBMPA	(47)
	Isocratic/ACN-H_2O, CH_3COONH_4	ESI	Reference samples	iPMPA, PinMPA, CHMPA, EMPA, iBMPA	(48)
	Isocratic/ACN-H_2O, CH_3COONH_4	ESI	Urine	iPMPA, PinMPA, CHMPA, EMPA, iBMPA	(49)
	Isocratic/ACN-H_2O, $HCOONH_4$	ESI	Urine	iPMPA	(44)
	Gradient/ACN-H_2O, CH_3COOH	ESI	Blood serum and urine	iPMPA, PinMPA, CHMPA, EMPA	(50)
	Isocratic/ACN-H_2O, CH_3COONH_4	ESI	Blood and blood serum	iPMPA, PinMPA, CHMPA, EMPA, iBMPA	(51)
	Precolumn derivatization/gradient, ACN-H_2O, CH_3COONH_4	ESI	Dust and environmental Samples	MPA, iPMPA, PinMPA, EMPA, iBMPA	(52)
	Gradient/ACN-H_2O, CH_3COONH_4	ESI	Standards	iMPA, PinMPA, CHMPA, EMPA, iBMPA	(53)

Notes: ACN, acetonitrile; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; HILIC, hydrophilic interaction chromatography; ICP, binductively coupled plasma; PGC, porous graphic carbon column; TS, thermospray ionization.

the high concentrations of acid in the mobile phase may reduce analyte response when utilizing ESI-MS. Additionally, Zhou and Hamburger (54) have reported that formic acid, in some cases, enhances the formation of $[M + H]^+$ ions for a range of compounds when using ESI-MS, and they have given a detailed discussion and explanation of the factors involved.

The alkyl methylphosphonic acids are especially challenging for retention on a reversed-phase chromatographic system. They are ionic and have extremely low pKa values (~2), and therefore, generally partition in the aqueous mobile phase and subsequently are not well retained. If positive ion mode is utilized, higher concentrations of acid in the mobile phase maybe required. MPA generally elutes near the dead volume of most systems, and this makes its detection and quantification problematic. Matrix effects can greatly affect the ionization of MPA and cause significant loss of its response. Analytical methods used for fairly simple sample matrices, such as environmental wipe samples, have been used to quantify MPA. For example, Willison (36) developed a method to wipe surfaces and evaluate the extraction recovery with an Ultra Performance Liquid Chromatography–MS/MS (UPLC–MS/MS) system to detect MPA as well as iPMPA, PinMPA and EMPA. This was a useful procedure for environmental surface testing (vinyl tile, painted drywall, wood, laminate, galvanized steel and glass surfaces), and should be considered when performing analysis of non-complex sample matrices. Experiments performed in the NIOSH laboratory involving urine analysis determined that MPA was not easily retained chromatographically and, thus, not immune from matrix effect problems. Therefore, in general, more extensive sample preparation before employment of the chromatographic methodology has often proven necessary for many of the alkyl methylphosphonic acid methods as reported in the literature.

One exception to a thorough sample pretreatment for analysis of these compounds was reported by Rodin *et al.* (22). This "dilute-and-shoot" method utilized a reversed-phase separation of iPMPA, PinMPA, EMPA and iBMPA from urine, and it should be noted that MPA and CHMPA were not targeted analytes. This separation was achieved using a gradient with mobile phases consisting of 0.5% formic acid in water and acetonitrile. The gradient was initiated at 98% with the water formic acid solution and 2% acetonitrile. Urine is an extremely complicated matrix and often requires special care for method ruggedness. Also, it should be noted that not all C18 columns and packing materials perform alike. Experiments were conducted in this NIOSH laboratory and demonstrated a separation of iPMPA, PinMPA, CHMPA, EMPA and iBMPA using a Waters Cortecs C18 3 × 150 mm column with 2.7 µm particle size (Figure 2). The Cortecs column was found to be the only one capable of chromatographically separating CHMPA and PinMPA of the different manufacturers' columns tested using gradients. The optimized gradient utilized 0.6% (v/v) formic acid in water and acetonitrile mixtures. The 0.6% level in the mobile phase was found to be the minimum level to give good peak shape for the analytes; levels higher than 0.8% tended to suppress the signal response of the analytes. CHMPA and PinMPA could certainly be separated by mass signals, but a chromatographic separation of the analytes was desired in this study. The optimized gradient in the chromatogram shown in Figure 2 used a mobile phase A consisting of 3/96.4/0.6% (v/v/v) acetonitrile/water/formic acid and mobile phase B consisting of 75/24.4/0.6% (v/v/v) acetonitrile/water/formic acid. The separation system consisted of both a mobile phase composition and flow rate gradient, the initial flow of 0.2 mL/min and 100% mobile phase A for 4.5 min, ramping up to a flow of 0.25 mL/min and 65% mobile phase B for 4 min and then to 90% B for 2.5 min and increasing the flow to 0.35 mL/min and mobile phase composition to 100% B for the final 4 min.

The use of reversed-phase ion-pair chromatography for the analysis of the alkyl methylphosphonic acids has been used as a retention and separation strategy. Simple reversed-phase HPLC only uses mobile phases that are of mostly aqueous content and contain a portion of organic modifier. The separation of analytes is performed using a stationary phase that has a surface less polar than the mobile phase. In reversed-phase ion-pair chromatography, a counter-ion is added to the mobile phase, and a secondary chemical equilibrium of the ion-pair formation is used to control retention and selectivity. One of the most involved studies using ion-pair chromatography was done by Wils *et al.* (27). Various tetra ammonium salts were evaluated using a C18 column to separate MPA, iPMPA, PinMPA, CHMPA and EMPA. Richardson, et al. (25) used an Alltima C8 3.2 × 150 mm column with a 5 µm particle size and an ICP-MS detector to generate the chromatogram displayed in Figure 3. In that study, myristyl trimethylammonium bromide was used as the ion-pair reagent with a 98/2 (v/v) water/methanol mobile phase. B'Hymer and Cheever (16) also explored ion-pair separations using hepatfluorobutyric acid. Reversed-phase ion-pair systems have generally attempted to gain better retention of MPA to move it away from the void volume of the chromatographic system and possible interferences or ion suppression.

Capillary and ultra-high performance liquid chromatography

The use of capillaries packed with stationary phase has been employed in the past for analysis of alky methylphosphonic acids and has been reported in the literature (38–40). The general purpose for using capillary columns was to increase theoretical plates. The more recent advancement into this line of increasing theoretical plate count is with ultra-high performance liquid chromatography (UPLC). This has come about from the availability smaller particle size column packings and the HPLC pump manufacturer's ability to create pumping systems suited to withstand the high back pressure level required to perform this technique. Standard HPLC pumping systems have historically been capable of maximum pressure levels of ~6,000 psi while UPLC systems are designed to handle pressures in excess of 15,000 psi.

The real advantage of UPLC is in the increase in theoretical plates for the separation analytes. Figure 4 displays a chromatogram obtained using a Cortecs C18 3.0 × 150 mm column with 1.7 µm particle size and the same mobile phase and gradient as described for the chromatogram displayed in Figure 2. The separation of iPMPA, PinMPA, CHMPA, EMPA and iBMPA is similar, but with slight improvements in peak shape and slight increases in peak theoretical plate count for the later eluting analytes iBMPA, CHMPA and PinMPA.

Willison (36) performed a similar study between standard HPLC versus UPLC with the objective being to reduce chromatographic run time and chromatograms are displayed in Figure 5. The HPLC system utilized an Atlantic dC18 column, 2.1 × 150 mm with 3 µm particle size and a flow rate of 0.3 mL/min. A multistep gradient was used with 0.2% (v/v) formic acid in water and acetonitrile over 12 minutes to reach a 70% acetonitrile composition. The UPLC system utilized an Acquity BEH C18 2.1 × 50 mm column with 1.7 µm particle size and similar mobile phase at a flow rate of 0.5 mL/min. The gradient time was only 2.5 min to reach a 70% acetonitrile composition.

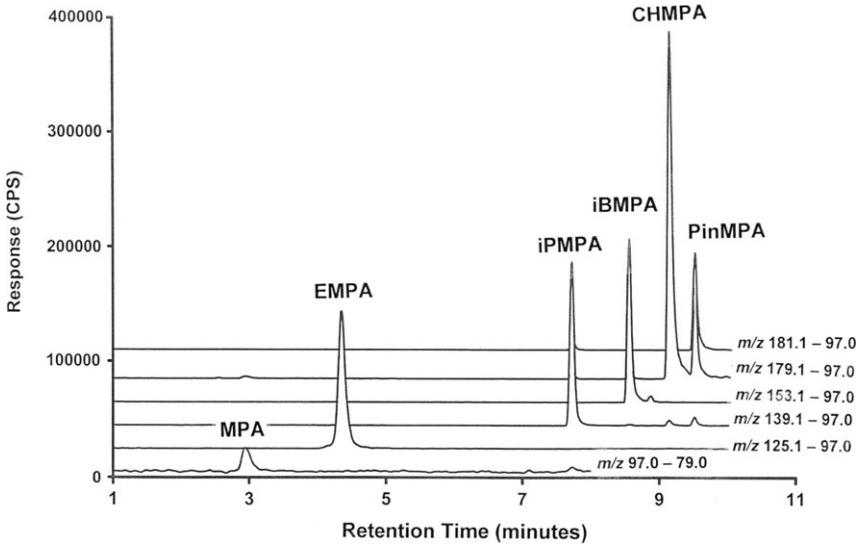


Figure 2. Gradient HPLC reversed-phase system using a column with a 2.7 μm particle size; all the alkyl methylphosphonic acids are at a 20 ng/mL concentration. Chromatographic conditions are listed in the text (chromatogram from author's collection).

Again, the UPLC system was designed to reduce chromatographic run time. The samples for this study were environmental wipes, not complicated biological matrices, and they were relatively free from interferences or ion suppression problems.

Hydrophilic interaction liquid chromatography

A more recent trend in the analysis for alkyl methylphosphonic acids is the use of hydrophilic interaction liquid chromatography (HILIC). Although reversed-phase chromatography is common and fully compatible with electrospray ionization interfaces with MS, it has the major limitation in the lack of retention of the hydrophilic, ionic alkyl methylphosphonic acids. HILIC chromatography is variation of normal phase chromatography in which there is a polar stationary phase and a high organic content to the mobile phase. HILIC has some important advantages over normal phase; the most important is that of the column being acceptable for the use of higher aqueous content mobile phases which would dissolve the silica packing material of the older normal phase columns. Hemstrom and Irgum (55) have described the HILIC mechanism in detail, while Nguyen and Schug (56) have described the advantages of HILIC when combined with ESI-MS detection. HILIC has an advantage in the analysis of the alkyl methylphosphonic acids in organic matricies; it is well suited for the direct analysis (dilute-and-shoot) in environmental samples prepared by SPE where there is already a high organic content to the sample to be injected into the chromatographic system. Protein precipitation typically performed in the analysis or blood, plasma or serum is typically done in with organic matricies. Generally, acetonitrile is utilized as the organic component in the mobile phases of HILIC chromatographic methods. In its use for the detection of the alkyl methylphosphonic acids, MPA generally does not chromatograph well in HILIC systems as it tends to be retained too long, and the majority of the methods reported in the literature detect and quantitate the other alkyl methylphosphonic acids. In the sole example of a normal phase system reported in the literature, MPA was eluted by a mobile phase having an aqueous content of 50% (57).

Mawhinney *et al.* (48) utilized HILIC chromatography in order to better optimize the electrospray conditions to give the best signal and signal-to-noise (S/N) ratios for the alkyl methylphosphonic acid analytes. In Mawhinney's study (48), it was reported that increasing signal intensity followed proportionally with increasing acetonitrile content up to mobile phase compositions of ~80%. When approaching 100% acetonitrile content, the trend reversed itself owing to unstable ESI conditions and a low concentration of suitable proton acceptors needed to form anionic analyte species (56, 58).

As can be seen in Table II, most HILIC systems reported in the literature are isocratic. Gradient experimentation performed in the NIOSH laboratory showed a rather long re-equilibration time when using HILIC chromatography, which is also a general trait of regular normal phase chromatography. A typical chromatogram using an Atlantis HILIC Silica 3 um column (150 × 2.1 mm) and a mobile phase of 85/15 (v/v) acetonitrile/water at 10 mM ammonium acetate buffer is shown in Figure 6. There was one exception to isocratic separations; a gradient system was reported by O'Connor (53) in the separation of iPMPA, PinMPA, CHMPA, EMPA and iBMPA. This separation system reported a fast cyclic time of only 4 min.

Adsorption HPLC

In adsoption chromatography, analyte retention is assumed to be a displacement process (59). Although there are many different stationary phases for adsorption chromatography, porous graphitic carbon (PGC) is of interest for this review. PGC has very unique properties as a stationary phase and has been reviewed in the literature (60). For non-polar analytes, PGC behaves similarly to a strongly retentive alkyl-bonded stationary phase; however, its retention and selectivity behavior toward polar and structurally similar compounds is very much different. Retention of a molecule with a polar group in reversed-phase systems will generally reduce retention times, while PGC systems may not be as affected or may actually increase retention owing to a polar retention effect with the stationary phase. This, along with other complex retention mechanisms, and PGC's ability to use more extreme pH conditions can make it very useful for difficult separation problems. Mercier *et al.* (45) did employ a porous graphitic carbon column to separate multiple alkyl phosphonic acids; a chromatogram of this work is displayed in Figure 7. This system utilized a Hypercarb two 2.1 × 150 mm column with a particle size of 7 μm and a gradient with a mobile phase consisting of 0.1% trifluoroacetic acid and

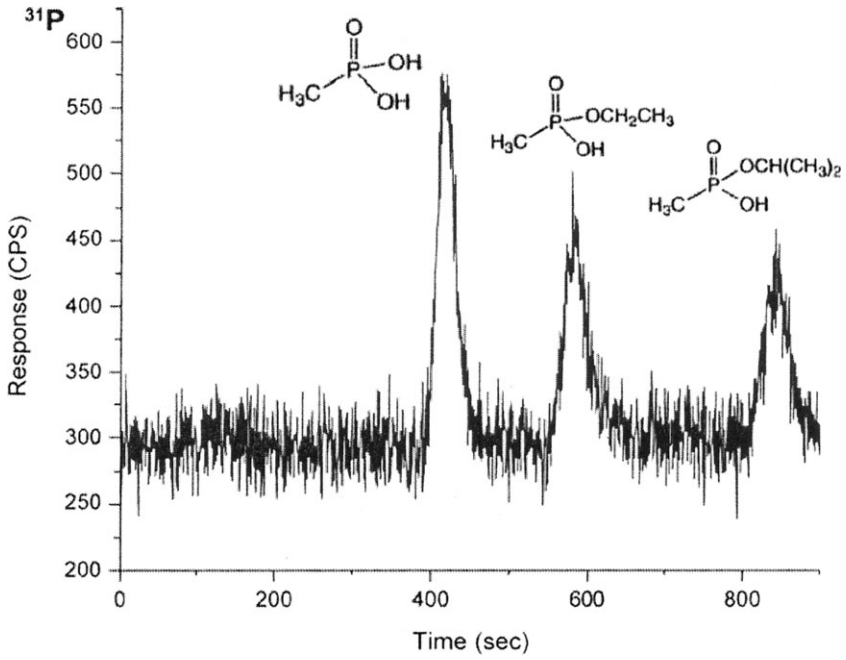


Figure 3. Separation of 100 ng/mL mixture of MPA, EMPA and iPMPA with ICP-MS detection. From reference 25, page 399, reprinted with permission of the Royal Society of Chemistry, copyright 2006.

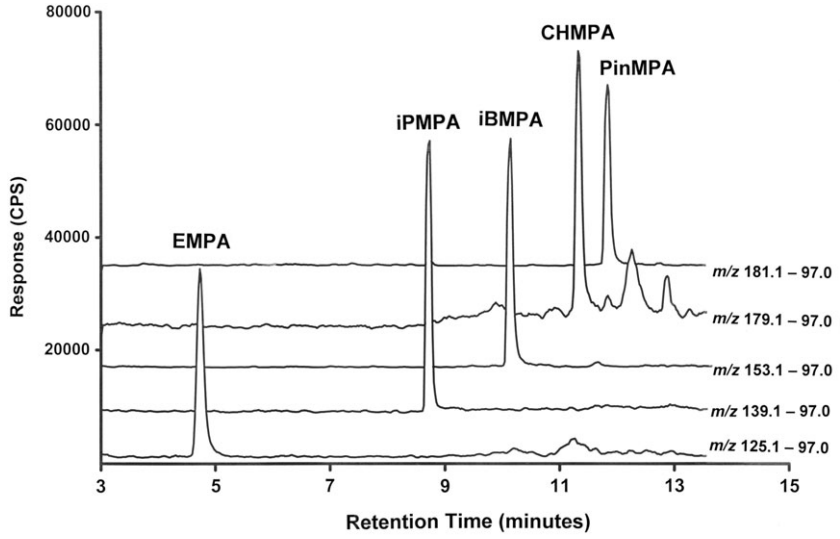


Figure 4. Gradient HPLC reversed-phase system using a column with a 1.7 μm particle size; all the alkyl methylphosphonic acids are at a 20 ng/mL concentration. Chromatographic conditions are listed in the text (Chromatogram from author's collection).

acetonitrile as the organic modifier. In Mercier's *et al.* (45) evaluation of this separation, trifluoroacetic acid was found to be the optimal acid to effect chromatographic separation of the alkyl methylphosphonic acid analytes when compared to other carboxylic acids. Tak *et al.* (46) also utilized PGC columns in the analysis of these acids.

Ion-exchange HPLC

Ion-exchange chromatography uses the mechanism of exchange equilibrium between a stationary phase containing surface ions and the oppositely charged ions contained within the mobile phase. Analyte counter ions compete for the ions on the surface of the stationary phase, and the relative affinities of the solute ions determine the extent of retention of the analytes. Owing to the ionic nature of the alkyl methylphosphonic acids, this would be a logically explored chromatographic system for the alkyl methylphosphonic acids. Noort *et al.* (42) was one example using an anion-exchange column to determine the level of iPMPA in blood serum from Japanese victims of 1995's Sarin terrorist attack.

Mixed-mode HPLC

Mixed-mode HPLC, that is, a stationary phase consisting of a reversed-phase stationary phase with embedded ion-pairing groups,

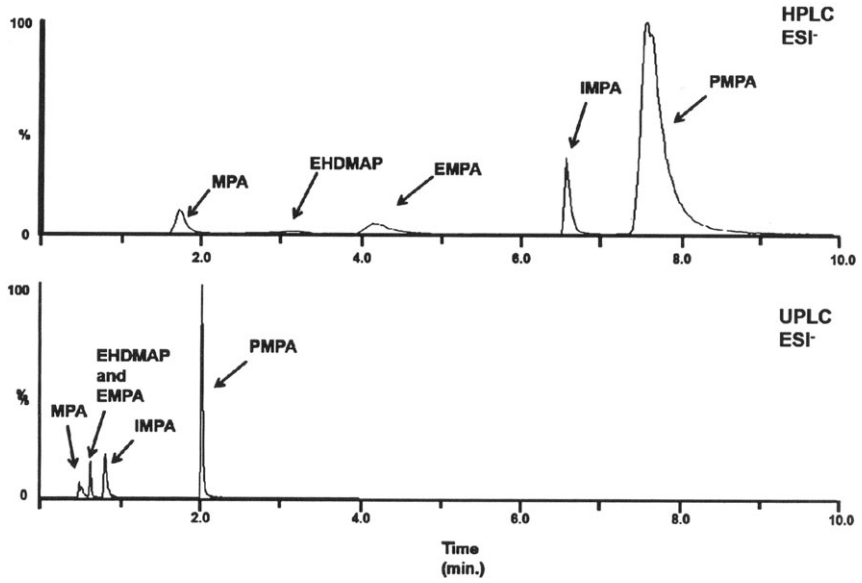


Figure 5. Reversed-phase system comparison of HPLC versus UPLC with emphasis on reducing chromatographic run time. EHDMAP is ethyl hydrogen dimethylamidophosphate and is a degradation product of the agent GA. From reference (36), page1037, and is not subject of U.S. copyright of the American Chemical Society.

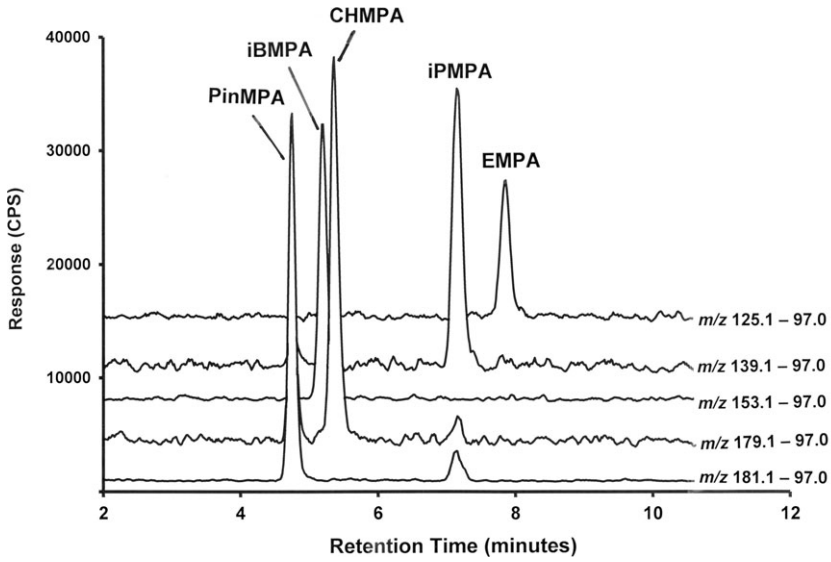


Figure 6. Isocratic HILIC system; all the alkyl methylphosphonic acids are at a 25 ng/mL concentration. Chromatographic conditions are listed in the text (Chromatogram from author's collection).

has been used by Riches (44) to determine MPA and iPMPA from spiked urine using an isocratic HPLC system. Mixed-mode columns have the advantage of improving the retention of acidic compounds when basic ion-pair groups are included in the stationary phase by an anion-exchange mechanism. Therefore, the alkyl methylphosphonic acid analytes are well suited for this type of separation system.

Conclusions and Future Trends

HPLC–MS has provided a powerful analysis tool for the alkyl methylphosphonic acids. HPLC–MS has allowed test methods to be produced that eliminate the need for chemical derivatization necessary for gas chromatographic analysis and can reduce some pre-sample cleanup treatments. MS–MS offers the greatest level of test method specificity for analysis and will likely be the predominant technique utilized for many years to come. The alkyl methylphosphonic acids offer many chromatographic challenges using reversed-phase systems, but the advances in stationary phases and the further experimentation with HILIC, mixed-mode and porous graphitic carbon systems will undoubtedly lead to improved separation methods. These improved methods will ease some of the concern for preparedness for detection and rapid response to potential releases of nerve agents.

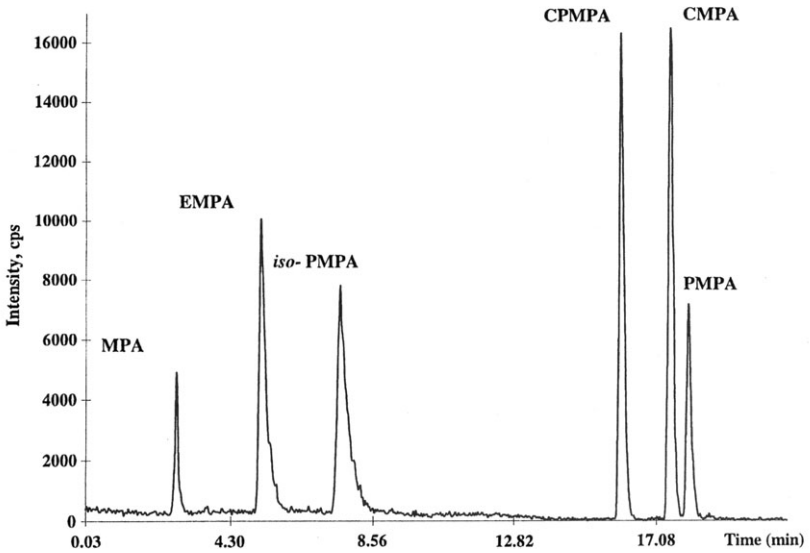


Figure 7. Gradient HPLC adsorption HPLC column displaying excellent separation of the alkyl methylphosphonic acids. From reference 45 reprinted with permission of Elsiever, copyright 1999.

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Conflict of interest statement

The author hereby reports that I have no conflict of interest with the material reported in the article. The author alone is responsible for the content and writing of this article.

Disclaimers

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health (NIOSH) or the Centers for Disease Control and Prevention (CDC). Mention of company names and/or products does not constitute endorsement by NIOSH.

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