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Gas chromatography-mass spectrometry with spiral large-volume injection for determination of fluoridated phosphonates produced by fluoride-mediated regeneration of nerve agent adduct in human serum

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ABSTRACT

A sensitive method for determination of fluoridated phosphonates produced by fluoride-mediated regeneration of nerve agent adduct in human serum was developed using gas chromatography-mass spectrometry (GC—MS) with large-volume injection. The GC injection was administered using stomach-type spiral injector (LVI, AiSTI SCIENCE) enabling introduction of only target compounds from 50 μL ethyl acetate extract after purging the solvent. For GC—MS analysis of sarin (GB), 670 times higher sensitivity, based on limit of detection (LOD, S/N = 3, on extracted ion chromatogram (EIC) at *m/z* 99), was achieved using this injection (50 μL) compared to that achieved using 1 μL split injection (ratio 20:1). Ethyl (EtGB), isopropyl (GB), n-propyl (nPrGB), isobutyl (iBuGB), pinacolyl (GD), cyclohexyl (GF) methylphosphonofluoridates, and *O*-ethyl *N, N*-dimethylphosphoramidofluoridate (GAF) were detected with low LOD (15–75 pg/mL) and sharp peak shapes (high practical plate number (defined as 5.54 x $(t_R/W_h)^2$, where t_R is the retention time and W_h is the bandwidth at half-height): 1100000–2400000) in GC—MS using a polar separation column, electron ionization, and quadruple mass analyzer. During the analysis of fluoridated phosphonate-spiked ethyl acetate extract of solid phase extraction (SPE, Bond Elut NEXUS) from fluoride-mediated regeneration of blank human plasma, LOD (on EIC at *m/z* 99 except for GAF (*m/z* 126)) were 25–140 pg/mL with sharp peak shapes. The reaction recoveries in fluoride-mediated regeneration of plasma, which was inhibited by GB, GD, GA, GF, VX, and Russian VX (10 ng/mL), were 49–114% except for GD (10%). The concentration levels of 0.3–1 ng/mL of nerve agents in plasma could be determined.

1. Introduction

Nerve agents, a type of chemical warfare agents [1] that manifest extreme toxicity towards humans via organophosphorus anti-cholinesterase action, are prohibited for usage, production, and storage [2], and are regarded as preferentially detection-targeted compounds. In the Matsumoto attack in 1994 and the Tokyo Subway system attacks in 1995, the cult group Aum Shinrikyo used sarin (GB) gas against defenseless people, causing many deaths and injuries [3]. In the Syrian war, many citizens were killed by GB bombs [4,5]. In 2017, Kim Jong Nam was assassinated by VX at Kuala Lumpur international airport in Malaysia [6]. In suspected and real chemical warfare/terrorism [7], besides on-site detection [8] performed by first responders and laboratory analysis of environmental and field samples [9], analysis of biological specimens [10,11] have been performed to identify causative toxic substances and to verify the exposure of the casualties to nerve agents. In forensic investigation on Tokyo subway GB gas attack, we did not detect GB but a product obtained from GB hydrolysis, that is, isopropyl methylphosphonic acid (IMPA), by gas chromatography-mass spectrometry (GC—MS) after *tert.*-butyldimethylsilyl (TBDMS) derivatization [12,13], from the blood samples of fatal casualties [3]. However, no such hydrolysis product

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could be detected from the samples of nonfatal casualties. Only the decrease in activities of red blood cell (RBC) acetylcholinesterase (AChE) and serum butyrylcholinesterase (BuChE) [14] was indicative of anti-cholinesterase agent exposure and poisoning [3].

In a human body, a nerve agent gets incorporated into the blood stream, and at first, serum BuChE (4.6 μg (0.054 nmol)/mL plasma [15]) and RBC AChE (0.0022 nmol/mL [16,17]), containing high affinity binding sites for nerve agents, adsorb the nerve agent via an irreversible inhibition mechanism (serine adduct formation). Blood serum BuChE level is assumed to be one hundred-fold higher than that of RBC AChE, and so BuChE is the major binding site for a nerve agent. Since the total volume of blood is about 6 L per human body, and the volumetric ratio of plasma and RBC in blood is about 1:1, it is estimated that there are about 160 nmol BuChE active sites in the human body. Therefore, when a nerve agent invades the human body, less than 160 nmol nerve agent can be presumed to be preferentially adsorbed by plasma BuChE. When more than 160 nmol nerve agent is to be adsorbed, the remaining agent that is not adsorbed by plasma BuChE is transferred to the other organs. The central nervous system is the main target of a nerve agent. Nerve agent is also partially detoxified by paraoxonase, a low affinity enzyme [18] present in blood plasma, or hydrolyzed spontaneously, and finally excreted via the urinary gland. Nerve agents are also adsorbed by albumin, low affinity binding site to make tyrosine adduct. [19]. Since the fatal level of GB is about 1.2 mg (8600 nmol), where fatal dosage is 150 mg min/m^3 [8] and respiratory volume during 1 min of respiration contains about 8 L air, when more than 8600 nmol of the nerve agent is adsorbed by the human body, the person immediately dies, and the hydrolyzed compounds of the nerve agent are not excreted via the urinary gland. The exposure level that manifests toxicity but not fatality can be expected to be less than one-tenth of the fatal level, and thus, it may be less than 1000 nmol.

Biological monitoring methods for nerve agent exposure have been developed for detecting specific biomarkers [11] of not only the hydrolyzed products but also the protein adducts. The latter targets, that is, protein adducts, could be detected after long periods of time owing to a long half-life of blood proteins, and thus low levels of exposure could also be verified. Basically, these methods should be highly sensitive to detect around one ng/mL levels of nerve agents in blood. The following two analytical methods have been developed: (1) liquid chromatography (LC)-MS for adduct peptides after protease digestion of the adduct proteins [20–23], (2) GC—MS of fluoridated phosphonates produced by fluoride-mediated regeneration of nerve agent adduct. The latter method was developed by Pollhuijs et al [25], and applied to the analysis of casualty blood samples from the Matsumoto GB attack. Initially, the target adduct was plasma BuChE GB adduct, but later the target nerve agents were expanded [25–28]. In addition, the adduct proteins were expanded to RBC AChE [25,29,30] and albumin (tyrosine adduct) [19]. Technically, plasma samples were treated with concentrated potassium fluoride under acidic conditions, applied to Sep-Pak C18 SPE cartridge, and then the resulting fluoridated phosphonates were eluted with ethyl acetate. After water elimination by treatment with dry ice acetone, the extract was analyzed by GC using nitrogen-phosphorus detection (NPD) [24]. Later, although the regeneration conditions and SPE elution solvent of ethyl acetate were not changed, some modifications were made, including changing SPE cartridge to highly permeable Bond Elut NEXUS [31], and altering GC detection hardware and GC injection to raise the sensitivity [31–34]. Basically, instead of either NPD or pulsed flame photometric detection [32], electron ionization quadrupole (Q-pole) MS detection was used, and its sensitivity was raised by adopting chemical ionization (CI) [4,26–31], high resolution MS [27,33,34] or tandem MS [5,25,30]. Besides, increase in GC injection volume raised the sensitivity, for which splitless injection [5,25,27,30,33], solid phase thermal-desorption-cooled injection (TD-CI) [31,32,35], or programed temperature vaporization (PTV) injection [26,29,34] were examined. Alternatively, the target compounds eluted by chloroform from SPE cartridge were concentrated using nitrogen gas blow evaporation with ethyl acetate [5,33,34].

To cope with biological monitoring of low level nerve agent exposure during fluoride-mediated regeneration, GC injection with large-volume of solid phase extract is efficient. The methods of prior researches, which include off-line thermal-desorption-cooled injection, PTV injection, or nitrogen gas blow evaporation with chloroform, seemed tedious, and owing to possible evaporation of volatile fluoridated phosphonates, reproducible quantification cannot be anticipated. Taking into account the highly volatile nature of fluoridated phosphonates (boiling point of GB: 158 °C), large-volume injection (LVI) of the solid phase extract without evaporative loss is attractive, and also, the procedure should be rather simple with good reproducibility. Spiral LVI technology was developed by AiSTi SCIENCE (Wakayama, Japan), and now commercial LVI devices have been introduced in analytical chemistry areas such as food and environmental sciences. For example, compounds with bad odor and taste in tap water, such as geosmine, were successfully analyzed by GC–MS with LVI after SPE [36]. Schematics of the spiral LVI device are shown in Fig. 1. This injector can be installed in any kind of GC machine. It consists of stomach-type glass line. First, large-volume of the sample solvent is injected, and trapped at the bottom of the stomach port at low temperature. Then, the solvent is discarded into the purge line at higher temperature and large carrier gas flow. Then, the target compounds remaining at the bottom are injected to the GC column with even higher temperature. Finally, the remaining compounds with rather high boiling temperatures are purged with high temperature and large carrier gas flow, and the injector is thermally cleaned to be ready for next analysis. The temperature and gas flow control enables selective and reproducible injection of analytes with solvent purging. The analytes for which the boiling points were 20 °C higher than that of the solvent can be applied. The operation is automatic without off-line sample procedure. The PTV injection is a prototype LVI, which the temperature-controlled sample liner is positioned between the injection port and GC column vertically [37]. This vertical liner structure may limit the applied sample volume, compared to spiral LVI system.

In this paper, spiral LVI is adopted for sensitive determination of seven volatile fluoridated phosphonates, and utility of this system is confirmed for sensitive identification of plasma adduct with nerve agents. Reaction scheme of fluoride-mediated regeneration of six nerve agents is shown in Fig. 2. The peaks of the fluoridated phosphonates showed high resolution and detection sensitivity with sub nanogram per milliliter level of detection. Thus, the method was successfully applied to plasma inhibited by low level of nerve agents.

2. Materials and methods

2.1. Reagent

The general chemicals used were of analytical grade. *O*-Isopropyl methylphosphonofluoridate (GB), *O*-pinacolyl methylphosphonofluoridate (Soman, GD), *O*-ethyl *N, N*-dimethylphosphoramidocyanidate (Tabun, GA), *O*-cyclohexyl methylphosphonofluoridate (cyclohexylsarin, GF), *O*-ethyl methylphosphonofluoridate (ethylsarin, EtGB), *O*-isobutyl methylphosphonofluoridate (isobutylsarin, iBuGB), *O*-ethyl S-*N, N*-diisopropylaminoethyl methylphosphonothiolate (VX), *O*-isobutyl S-*N, N*-diethylaminoethyl methylphosphonothiolate (Russian VX, RVX), and *O*-Ethyl *N, N*-dimethylphosphoramidofluoridate (fluo-

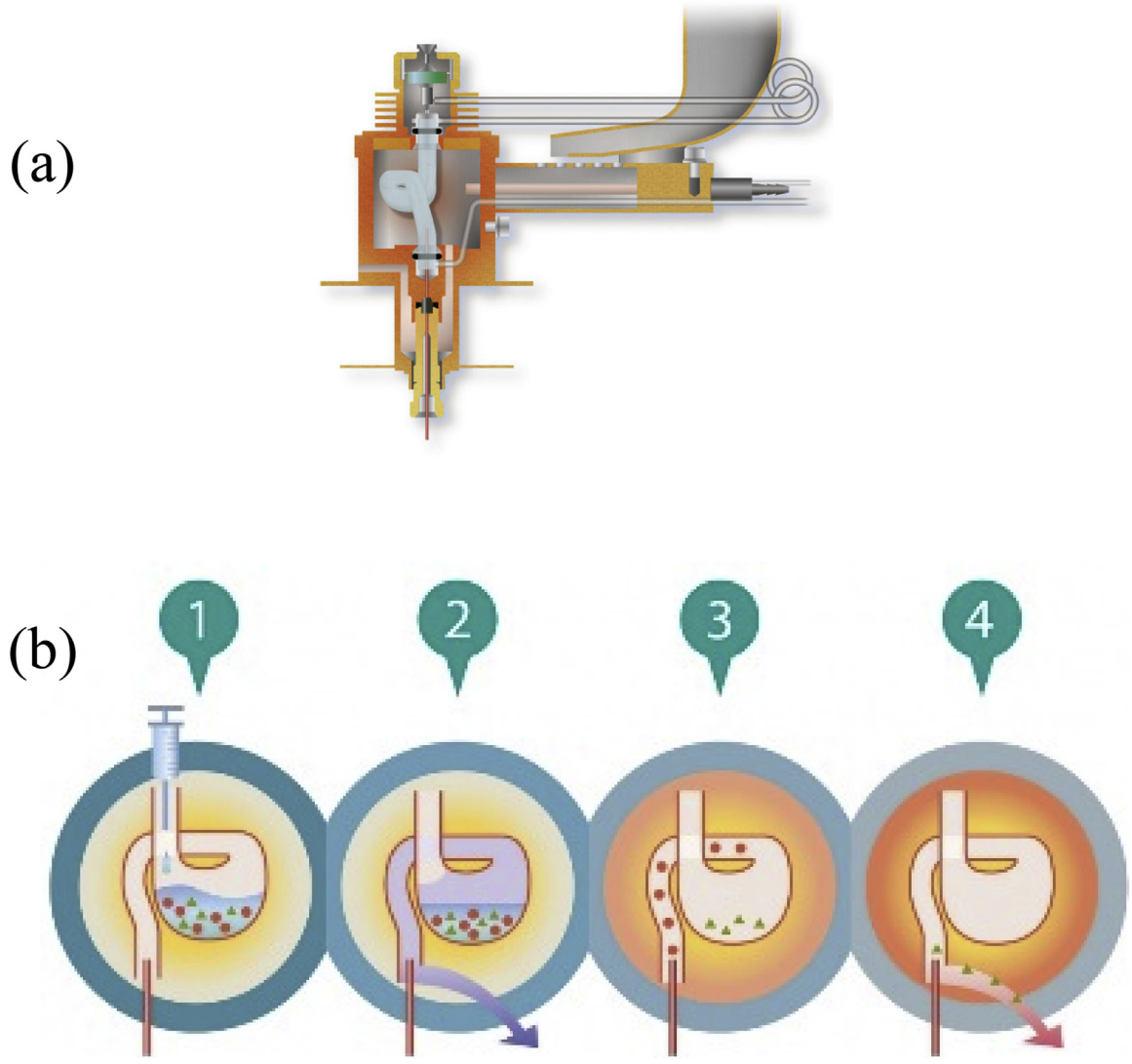

Fig. 1. Structure of spiral injector (a) and schematic diagram of large-volume injection (b). (1) Sample injection: solvent (blue) containing the analytes (circle) and nonanalytes (triangle) is retained at the bottom of the injector; (2) Solvent purging: the solvent is blown into the purge line; (3) Analyte introduction: analytes are introduced into the GC column; (d) Injector washing: the remaining high-boiling point compounds are discarded into the purge line and the injector is thermally cleaned.

rotabun, GAF) were synthesized in-house [38,39]. Their purities were evaluated to be more than 98% except for GAF (70%). Water used was purified by a MilliQ gradient A-10 system (Millipore, Redford, MA, USA).

2.2. Instrumentation

The LVI GC–MS system consisted of a 7890 series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a large-volume injector (LVI-S200, AiSTI SCIENCE, Wakayama, Japan), and a 5973 Q-pole mass selective detector (Agilent Technologies). Fifty microliters of ethyl acetate extract was injected into the injector automatically which was initially maintained at a temperature of 80 °C for 0.8 min, then the temperature was raised to 240 °C at the rate of 120 °C/min, and then to 290 °C at the rate of 50 °C/min. During the injection, helium was flown at 150 mL/min with solvent vent open for first 0.76 min. The GC–MS conditions were as follows: column, DB-5 (0.25 mm x 30 m, thickness, 0.25 μm), 40 °C for 4 min, 40–300 °C at 20 °C/min, helium 1.2 mL/min; ionization: electron ionization, ion source temperature: 230 °C; scan: 13 scan/s, *m/z*: 40∼350. Schematic diagram of the injection is shown in Fig. 1.

The split and splitless injections were performed using the same 7890 series gas chromatography injector coupled with a 5973 Q-pole mass selective detector. One microliter of ethyl acetate solution containing fluoridated phosphonates was injected into the split/splitless injector automatically, which was maintained at 250 °C with column flow of 0.7 mL in both split mode (ratio 20:1) and in splitless mode (after injection, the split flow was stopped for 2 min, and then raised to 50 mL). The GC–MS conditions were the same as the above except for the GC temperature program, which was changed to 40 °C for 1 min, followed by 40–290 °C at the rate of 20 °C/min, and holding the temperature of 290 °C for 5 min.

2.3. Reaction of human blood plasma with nerve agents

Human blood plasma (pool, heparinized) was obtained from Cosmo Bio Co., Ltd (Tokyo, Japan). One milliliter of control human plasma was incubated with 5 μL of diluted solution of nerve agent in isopropanol at 25 °C for 3 or 15 min.

BuChE activity was measured by modified Ellman's method [14]. Two hundred microliters of human plasma was mixed with 1 μL of isopropanol solution containing the nerve agent, and incubated at 25 °C for 10 min. The inhibited solution was diluted 50-fold with

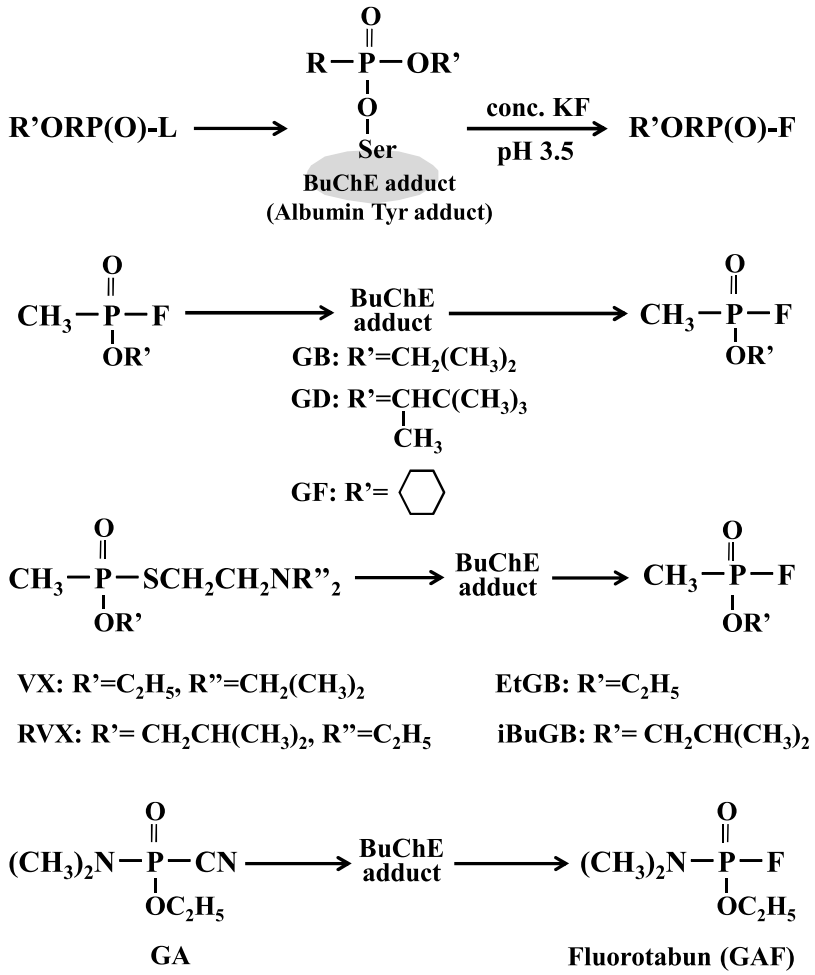

Fig. 2. Reaction of nerve agents with serum butyrylcholinesterase or albumin followed by fluoride-mediated regeneration of fluoridated phosphonates. The mark "L", "R" and "R'O" in "R'ORP(O)-L" mean leaving group, phosphorus-binding methyl or dimethylamino group, and alkoxyl group, respectively.

water, and 100 μL aliquot was preincubated with 3 mL of 0.25 mM 5, 5′-dithiobis(2-nitrobenzoic acid) in 5 mM sodium phosphate (pH 7.7) at 25 °C for 10 min. Then, BuChE reaction was triggered by addition of 40 μL of 156 mM butyrylthiocholine (BuTCh) iodide solution, and incubation at 25 °C for 15 min. The reaction was terminated by addition of 50 μL of 12 mM eserine sulfate. Absorbance at 405 nm was measured in a quartz cuvette (with 1 cm light-path length). Blank run was measured for the same reaction except for the preincubation with eserine. The molar absorption coefficient (ε) of the hydrolyzed substrate (thionitrobenzoic acid) was taken to be 13,500. One unit of BuChE is defined as the enzyme hydrolyzing 1 μmol of BuTCh per min. BuChE activity of control plasma is around 1 U/mL plasma.

2.4. Fluoride-mediated regeneration

The fluoride-mediated regeneration was performed as previously described by Degenhardt et al [31]. One milliliter of nerve agent-inhibited plasma was incubated with 3 mL of 0.189 M sodium acetate (pH 3.5) and 0.19 mL of 5.25 M potassium fluoride at 25 °C for 15 min. The reaction was terminated by the addition of 0.5 mL of 0.8 M sodium bicarbonate, and immediately the reaction mixture was applied to Bind Elut NEXAS (solid phase 200 mg/6 mL cartridge, Agilent Technologies (Santa Clara, CA, USA)), which was set on sample processing manifold (Vac Master-10, Biotage, Uppsala, Sweden), and equilibrated with 5 mL n-hexane, 5 mL ethyl acetate, and 10 mL water, and drawn under weak vacuum. The target compounds on the cartridge were eluted with 2 mL of ethyl acetate. Water from the ethyl acetate fraction was removed under dry ice-acetone, and the dehydrated fraction was subjected to GC–MS.

2.5. Safety considerations

Nerve agents are highly toxic. Therefore, protective clothing was worn and the chemical warfare agents (CWAs) were carefully handled within a fume hood and destroyed with sodium hypochlorite after analysis. The synthesis and use of GB, GD, GA, GF, EtGB, iBuGB, VX, and RVX was approved by the Ministry of Economy, Trade, and Industry of Japan [40].

3. Results and discussion

3.1. GC–MS with large-volume injection for fluoridated phosphonates in ethyl acetate solution

As solvent for GC injection, ethyl acetate (boiling point: 77 °C) was used because only ethyl acetate was used in study fluoride-mediated regeneration of nerve agent adduct in prior researches. Initially, taking into account the boiling points of the solvent and typical analyte, GB (boiling point: 158 °C), the initial injector temperature was set to be 80 °C, and initial temperature and retaining time for GC separation were set to be 40 °C and 4 min. Using GB as the analyte, the analytical conditions were optimized to provide acceptable peak shape, retention time, and highest peak area. The

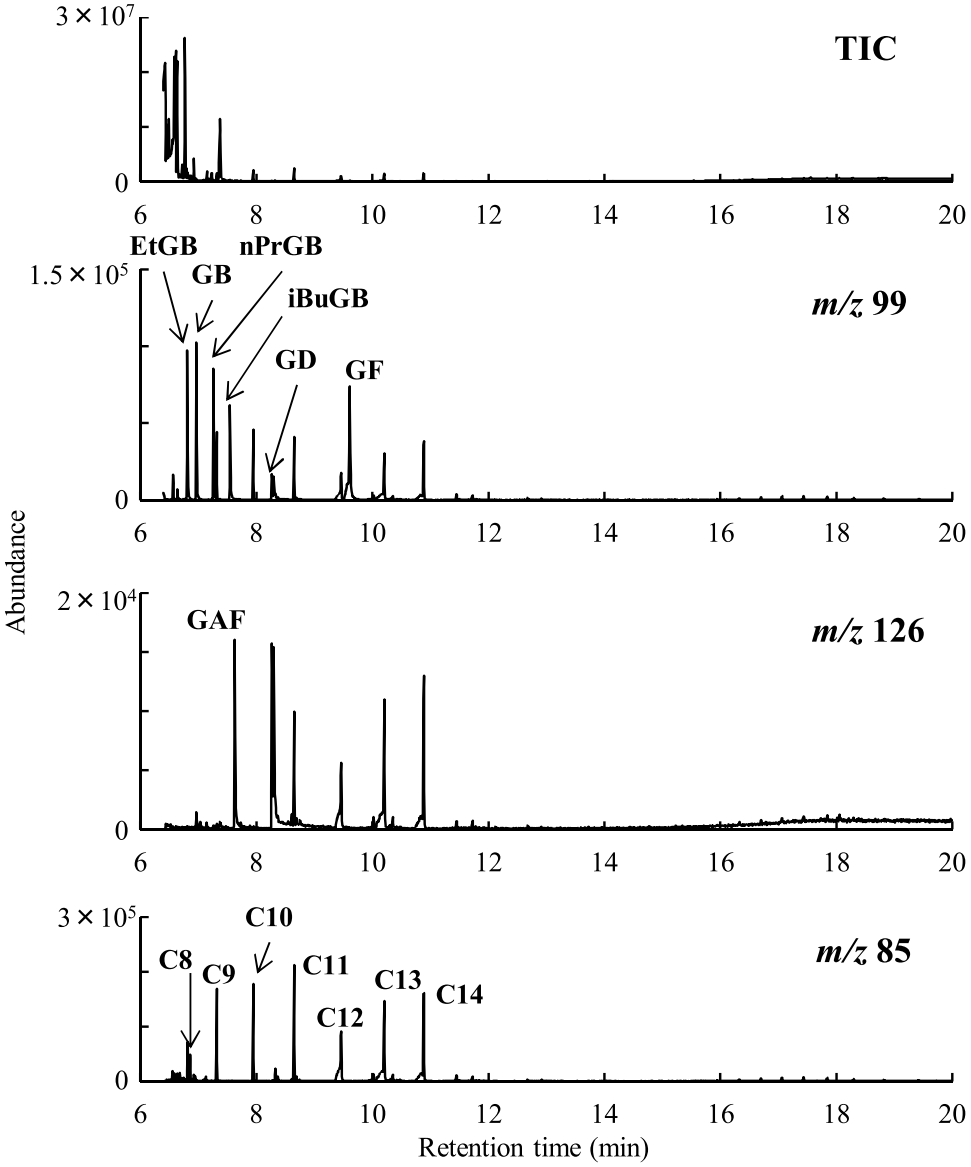

Fig. 3. Total ion and extracted ion (*m/z* 99, *m/z* 126, *m/z* 85) chromatograms of *O*-alkyl methyphosphonofluoridates and *O*-ethyl *N, N*-dimethylphosphoramidofluoridate (GAF). Ethyl acetate solution containing *O*-ethyl, *O*-isopropyl, *O*-n-propyl, *O*-isobutyl, *O*-pinacolyl, *O*-cyclohexyl methylphosphonofluoridates (EtGB, GB, nPrGB, iBuGB, GD, and GF; all 10 ng/mL), GAF (7 ng/mL), and n-alkanes (octane, nonane, decane, undecane, dodecane, tridecane, and tetradecane; 50 ng/mL) was injected into the GC–MS instrument with spiral large-volume injector.

conditions finally established are shown in Materials and Methods section. Seven analytes, which are EtGB, GB, nPrGB, iBuGB, GD, GF, and GAF, were analyzed by GC—MS with large-volume injection of 50 μL ethyl acetate solution. As shown in Fig. 3, the peaks for EtGB, GB, nPrGB, iBuGB, GAF, GD, and GF appeared in this order. These analytes could be well detected on extracted ion chromatograms of *m/z* 99 and *m/z* 126 as base peaks. Fig. S1 shows mass spectra of the analytes, which are identical with NIST library data spectra. Table 1 shows the determination performance of this GC—MS system. Quantitative ions and identifiable ions are shown with limits of detection (LODs), within-day repeatability shown as standard error (%), average slope values (peak area for each concentration) in calibration curves, their standard deviations (SD), average relative slope values compared to those for GB, SD for these values, number of the trials to obtain slope data, limit of identification obtained under the criteria of the allowable tolerance of the relative peak area intensity of identifiable ion to quantitative ion (obtained from Table S1), and number of the practical plate (NPP, defined as 5.54 x $(t_R/W_h)^2$, where t_R is the retention time and W_h is the bandwidth at half-height) of the analyte peaks, applied correspondingly from the theoretical plate number. Because the GC separation was performed by temperature programming, theoretical plate numbers could not be strictly calculated, but by comparing the NPP values, GC peak sharpness can be estimated. Calibration curves of the peak areas of the quantitative and identifiable ions against the concentrations provided linear or concave curves for 0 to 10 ng/mL concentrations of nerve agent (Fig. S2). The relative LODs for the quantitative and identifiable ions were almost similar to mass spectral peak intensities (Fig. S1). We compared the determination performance of this system by usual GC—MS, with either split or splitless injection. As shown in Table S2, split injection (ratio 20:1) provided sharp peaks of the target compounds with NPP values of 120,000–820,000. The LOD values were around 10 to 40 ng/mL level. Splitless injection provided low LOD values but not as low as expected. Especially, for more volatile analytes (GB–iBuGB) the peak resolution was low and the LOD values were

Table 1
Determination performance of gas chromatography-mass spectrometry with spiral large-volume injection for ethyl acetate solution containing *O*-alkyl methyphosphonofluoridates and *O*-ethyl *N, N*-dimethylphosphoramidofluoridate (GAF). The limits of detection (LODs) are obtained from the concentration showing signal to noise ratio of 3. Within-day repeatability is expressed as standard error value obtained from three measurements at 3 ng/mL fluoridated phosphonates. Slope value is obtained from standard curve of the quantitative ion peak areas against the concentrations (n = 12–17, except for nPrGB (n = 5)). The limit of identification (LOI) is expressed as the lowest concentration fulfilling relative ion peak intensity criteria as shown in Table S1. The practical plate number (NPP) is defined as 5.54 x $(t_R/W_h)^2$, where t_R is the retention time and W_h is the bandwidth at half-height.

Agent	*m/z*	LOD (pg/mL)	Within-day repeatability (%)	Slope[a] average	Slope SD	Relative slope average	Relative slope SD	Trial	LOI (pg/mL)	NPP
EtGB	99	15	6	87	69	0.805	0.343	14	30	2.38×10^6
	111	100								
GB	99	18	4.4	100	45	1		17	100	1.37×10^6
	125	42								
nPrGB	99	18	6.3	79	36	0.682	0.223	5	300	1.17×10^6
	111	120								
iBuGB	99	25	5.2	69	35	0.569	0.223	12	30	1.87×10^6
	112	34								
GD	99	75	3.9	28	15	0.307	0.121	16	300	1.57×10^6
	126	94								
GF	99	22	6.8	110	51	1.295	0.575	15	300	1.83×10^6
	67	190								
GAF	126	69	3.2	44	13	0.386	0.221	16	210	1.25×10^6
	155	140								

N: number of theoretical plates for agent peak.
[a] amount in pg/mL.

similar to those obtained after split injection, although the slope values were approximately 5-fold higher, which may be derived from lower peak resolution. EtGB could not be detected. In contrast, LVI mode provided considerably higher peak resolution and high detection sensitivity. By comparing the peak area values of the fluoridated phosphonates between LVI (50 μL injection) and splitless injection (1 μL injection) using the same GC—MS machine, the relative peak area values of LVI to splitless injection were 56 (GB), 75 (iBuGB), 51 (GD), 80 (GF) and 76 (GAF), respectively. Upon comparing the slope values and LOD of the LVI mode to those of the split mode (split ratio 20:1), the increased sensitivity ratios were 350–1600 and 440–1900. These ratios are compatible with the ratios of the injection volumes (50 μL vs 1 μL x 1/20). The carry-over of the target compounds was not observed, for there were no target peaks on the extracted ion chromatograms from the blank run after the standard sample run (50 μL injection of 10 ng/mL solution).

As for the retention times of the target compounds, considerable variation was observed. As shown in Table S3, the retention times of more volatile EtGB and GB deviated by about 1 min. For less volatile compounds, retention times were rather converged with small SD values. It may be possible because the stacking of the target compounds in the front of the separation GC column at 40 °C may be severely influenced by the condition of the concentrated solution at the stomach bottom in spiral injector. The timing of purging of the solvent is a delicate event. The retention index (RI) values against normal alkane are shown in Table S4. The RI values of the examined fluoridated phosphonates ranged from 790 to 1220. Not only within-day repeatability but also between-day repeatability was low. We can detect fluoridated phosphonates by the target analysis where initially the EIC with *m/z* of quantitative and identifiable ions are traced and then the elution position is ascertained by their RI values.

3.2. GC–MS with LVI for fluoridated phosphonates in plasma extract ethyl acetate solution

Before proceeding with the fluoride-mediated regeneration of nerve agent adducts, matrix effect of the plasma extract on GC–MS analysis with LVI was examined. It was speculated that not only MS detection but also GC separation derived from LVI were influenced by matrix components extracted from plasma treated by

Table 2
Determination performance of gas chromatography-mass spectrometry with spiral large-volume injection for ethyl acetate extract of solid phase extraction from fluoride-mediated regeneration reaction mixture of blank human serum containing *O*-alkyl methyphosphonofluoridates and *O*-ethyl *N,N*-dimethylphosphoramidofluoridate (GAF). LOI is measured for the lowest concentration fulfilling relative ion peak intensity criteria as shown in Table S2. NPP: number of practical plates for agent peak.

Agent	*m/z*	LOD g/mL	Within-day repeatability (%)	LOI pg/mL	NPP
EtGB	99	34	4.1	300	8.39×10^5
	111	140			
GB	99	33	3.1	100	1.00×10^6
	125	48			
nPrGB	99	45	2.9	300	1.11×10^6
	111	260			
iBuGB	99	58	4.7	100	1.16×10^6
	112	70			
GD	99	140	2.7	300	1.88×10^6
	126	110			
GF	99	25	4.4	1000	1.78×10^6
	67	320			
GAF	126	120	6.9	210	1.22×10^6
	155	190			

fluoridated regeneration reaction mixture. Fig. S3 shows typical GC traces for ethyl acetate extract of solid phase extraction from fluoridated regeneration reaction mixture of blank human serum, containing fluoridated phosphonates. On TIC, large components derived from the plasma were eluted mainly at a retention time later than 12 min, within the elution positions of high GC column temperature. Within the elution position of low GC column temperature, where fluoridated phosphonates appeared and the retention index was less than 1300, the matrix components exhibited less interference. However, severe interference could still be observed within these early retention times. With lower *m/z* ion extraction, background noise was higher. Table 2 shows the detection capacity of this GC–MS system, including the LOD, within-day repeatability shown as standard error (%), limit of identification obtained from Table S5, and NPT values. The LOD values were raised 1.1–2.5 fold compared to those for ethyl acetate solution, and the values of the limit of identification (LOI), which is expressed as the lowest concentration fulfilling relative ion peak intensity criteria as shown in Table S1, were either the same or raised 3.3- to 10-fold. Calibration curves of the peak areas for the quantitative and identifiable

Table 3
Reaction recovery during fluoride-mediated regeneration of fluoridated phosphonates from human plasma inhibited by middle level exposure to nerve agent. Human plasma was reacted with nerve agent (10 ng/mL) for 15 min (except for GD with which the reaction time was 3 min) at 25 °C, and fluoride-mediated regeneration was performed to produce the corresponding phosphonofluoridates. The net production quantity is obtained using the matrix calibration curves or by compensation for plasma extract matrix effect.

Inhibitory agent	VX	GB	RVX	GD	GD	GF	GA
Regenerated agent	EtGB	GB	iBuGB	GD	GD	GF	GAF
Reaction time (min)	15	15	15	15	3	15	15
Trial	5	12	4	2	3	6	5
Recovery (%)	59.2	49.4	114.4	0	10.5	52.8	68.2
SD (%)	15.8	11.1	27.9		6.3	17.3	18.3

ions against the concentrations provided linear or concave curves for concentration range of 0 to 10 ng/mL (Fig. S4). The yields of the peak areas for the quantitative and identifiable ions in plasma extract matrix compared to those in ethyl acetate solution were not constant, but rather deviated, depending on concentrations being examined, as shown in Fig. S5. As were the longer the retention time of fluoridated phosphonates, the relative yields were higher. The carry-over of the target compounds from the regenerated reaction extract was not observed, just the same as the standard solution.

From the LOD values based on the quantitative ion tracing shown in Table 2 and the reaction recovery values shown in Table 3, we could speculate the detection sensitivity of plasma protein adducts which were detected as regenerated fluoridated phosphonates. For GB, GF, GA, VX, and RVX, the LOD values were 130, 90, 350, 230, and 200 pg/mL plasma, respectively. The earlier regeneration method adopting GC-NPD yielded 200 pg GB/mL blood [24]. Then, Jakubowski et al reported an LOD of 16 pg GB/mL RBC, by adopting 50 μL PTV and ammonia CI Q-pole mass analyzer with isotope dilution [29]. Degenhardt et al reported an LOD of 10 pg/mL plasma for GB, GF, GA, and VX, by adopting 100–400 μL TD-CI and ammonia CI Q-pole mass analyzer [31]. Byers et al reported an LOD of 20 ng VX/mL RBC, by adopting 1–3 μL splitless injection and ammonia CI triple stage Q-pole mass analyzer with isotope dilution [17]. Renner et al obtained 500 pg GD/mL plasma, by adopting 50 μL PTV injection and ammonia CI Q-pole mass analyzer with isotope dilution [26]. Holland et al reported an LOD of 5–16 pg/mL serum for GB, GD, GF GA, and VX, by adopting SPE solvent concentration, 2 μL splitless injection, and sector mass analyzer [33]. Van der Meer et al reported an LOD of 70 pg/mL plasma for GB, GA and VX, by adopting SPE solvent concentration, 100 μL PTV injection, and time-of flight mass analyzer [24]. Off-course, the regeneration efficiency depending on target compounds and biological samples influences the method sensitivity. However, two factors of GC injection volume, including injection efficiency and MS detection sensitivity, are more important in determining the method sensitivity. According to the varied methods, LOD values based on sample volume (mL) varied from 5 pg/mL to 20 ng/mL. The LOD values of our method fell in-between this range. Considering that MS detection of electron ionization Q-pole mass analyzer is not sensitive compared to high-resolution MS and tandem MS, spiral LVI system could considerably contribute in raising the method's sensitivity.

The retention times of the target compounds in plasma extract largely fluctuated between days (data not shown). Although their elution times were not the same as for those in matrix-free solution, the average relative retention times (ratio of those in plasma extract to those in matrix-free solution) were almost one. Identification of the target compounds can be achieved both by EIC tracing and RI confirmation. As shown in Table S6, the RI values against normal alkanes were converged which could be ascertained with RI SD values, and these values were almost the same as those obtained from ethyl acetate solution.

3.3. Fluoride-mediated regeneration of fluoridated phosphonates from nerve agent-inhibited human plasma

In fluoride-mediated regeneration GC—MS method, extremely high sensitivity is required in order to detect nanogram per microliter levels of ChE adduct from plasma of the casualties with low level exposure. The disadvantage of this method is the phenomenon of aging, that is, enzymatic hydrolysis of alkoxyl group in the irreversibly bound nerve agents catalyzed by active site histidine. Especially, aging rate is very high for GD adduct. Fluoride-mediated regeneration cannot be achieved for aged adducts. For plasma sample of the casualties with high level exposure, it is possible to detect nerve agents with rapid aging against ChE, using tyrosine adduct of albumin which does not show aging [19].

Control human plasma was incubated with nerve agents (<150 nM concentration), and the residual BuChE activity was measured (Fig, S5). As the nerve agent concentrations were increased to 50 nM, the BuChE activity was decreased with 1:1 molecular stoichiometric relationship. The inhibition curves for nerve agents showed similar curve pattern. Because all the nerve agents are strong inhibitors of BuChE activity with high k_i/K_I (k_i: inhibition reaction constant (min^{-1}), K_I: dissociation constant (M^{-1})), the nerve agents bind to active sites of BuChE irreversibly. As the concentration of nerve agents was further increased from 50 nM to 150 nM, inhibition curve became concave. It is expected that the BuChE active site concentration in this commercially available plasma sample was about 100 nM.

The control human plasma sample was incubated for 15 min (or 3 min for GD) with a nerve agent (10 ng/mL plasma) that was below the concentration of BuChE active site. Then, fluoride-mediated regeneration was performed. The regenerated fluoridated phosphonates, corresponding to the reacted inhibitory agents were evaluated, and their concentrations were calculated after compensating for the plasma matrix effect. Table 3 shows the reaction recovery values. The reaction recovery comprises of regeneration yield of inhibitory nerve agent, and recovery of the regenerated agent from the reaction mixture into final solid phase extract. From RVX incubated plasma, reaction recovery was almost 100%. From GD incubated plasma, no GD was detected, indicating rapid aging reaction of GD adduct. Instead, 10% GD was recovered from plasma incubated for 3 min with GD. Taking into account the aging speed of GD BuChE adduct, which is in the order of minutes, regeneration achieved for GD was reasonable. For other agents, about 50% of the corresponding fluoridated phosphonates were recovered. It is conceivable that in cases inhibited by VX and GB, the regeneration yields might be perfect but the extraction recoveries were low because of high volatility of regenerated EtGB and GB. On the other hand, in cases inhibited by GF and GA, the regeneration yield may be low owing to steric hindrance of the alkoxy groups but the extraction recoveries were almost 100%.

Since spiral LVI enables introduction of almost all the sample volume into GC column, the sensitivity is extraordinarily increased, but all the plasma extract components are introduced into ionization source of mass analyzer, and so the source becomes dirty after repeated injections, resulting in decrease of sensitivity. We cleaned the ion source after every 40–50 injections, which restored the mass spectrometric sensitivity. The necessary frequent cleaning of ion source is one drawback in LVI. If the back flushing device was installed to GC, we could protect the ion source from getting dirty by back flushing the late eluting components contained in the plasma extract, preventing introduction of these dirty components into GC and MS.

The control human plasma sample was incubated for 15 min (3 min for GD) with a nerve agent (1, 0.3 ng/mL plasma), which was expected to be below the inhibitor level occupying 5% of BuChE active sites. The determination result for 1 ng GB/mL plasma is

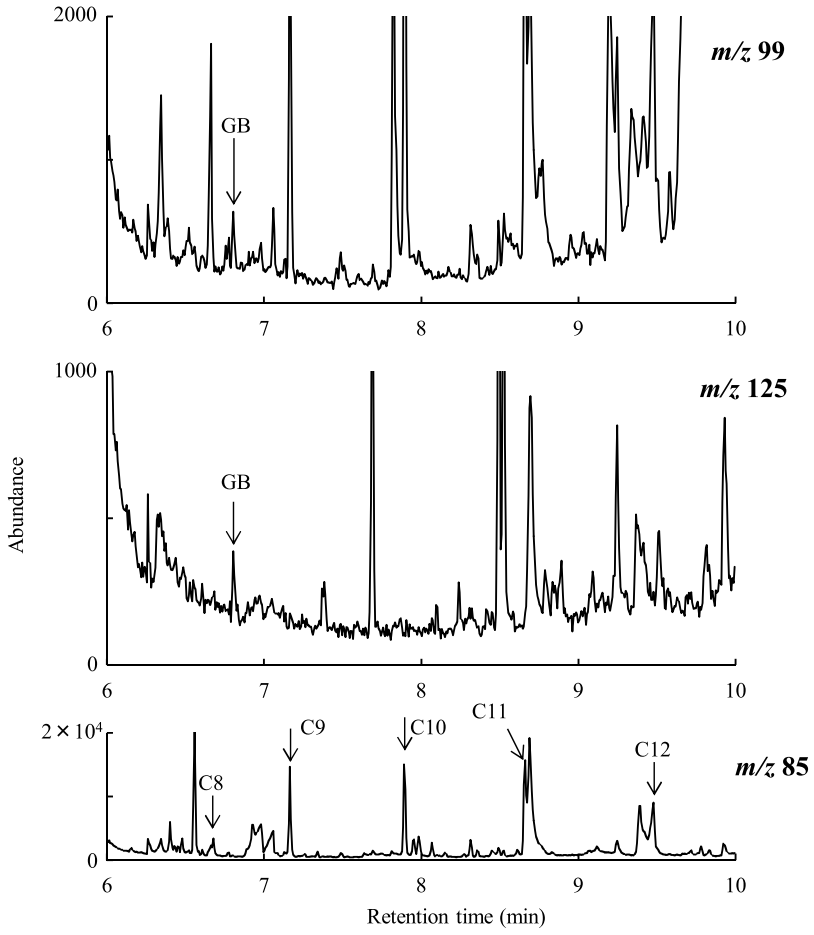

Fig. 4. Extracted ion (*m/z* 99, *m/z* 125, *m/z* 85) chromatograms of ethyl acetate extract of solid phase extraction from fluoride-mediated regeneration reaction mixture of human serum (1 mL) inhibited by sarin (GB, 15 min, 1 ng/mL). The retention time of GB ion is 6.80 min.

shown in Fig. 4. On EIC at both *m/z* 99 and *m/z* 125, a sharp peak at 6.80 min was observed, and the RI value was 826, determined by the comparison of normal alkane peaks with EIC at *m/z* 85. In addition, on EIC at both *m/z* 99 and *m/z* 111 (Fig. S7); *m/z* 99 and *m/z* 126 (Fig. S8); and *m/z* 99 and *m/z* 67 (Fig. S9), peaks at 7.11 min, 8.26 min, and 9.64 min, respectively, were observed for solid phase extract of fluoride-mediated regeneration from plasma samples inhibited by 1 ng/mL of nPrGB, GD, and GF, and the corresponding RI values were 890, 1048, and 1222 which were proven to belong to nPrGB, GD, and GF, respectively. Moreover, on EIC at both *m/z* 99 and *m/z* 111 (Fig. S10); *m/z* 99 and *m/z* 112 (Fig. S11); and *m/z* 126 and *m/z* 155 (Fig. S12), peaks at 6.54 min, 7.46 min, and 7.57 min, respectively, were observed from solid phase extract of fluoride-mediated regeneration from plasma samples inhibited by 1 ng/mL of VX, RVX, and GA, and the corresponding RI values were 784, 944, and 958, which were proven to belong to EtGB, iBuGB, and GAF, respectively. Table S7 shows the determination result of fluoridated phosphonates obtained from fluoride-mediated regeneration from the plasma inhibited by low levels of nerve agent (1, 0.3 ng/mL plasma). Reaction recovery was calculated tentatively although validation could not be achieved. The recovery values (Table S7) were comparable with those for the cases where the plasma was inhibited by 10 ng/mL concentration of nerve agent (Table 3). Identification (fulfillment of mass spectrometric criteria of relative peak area of the identifiable ion to quantitative ion) was also achieved for all the agents present in plasma at high levels (1 ng/mL), and for nPrGB and RVX present at low levels (0.3 ng/mL).

In this paper, single quadrupole mass analyzer was used as detector. Even though large-volumes can be introduced into a GC column, the LOD for fluoridated phosphonates was not low in this study, compared to that reported in the previous paper that provided sub picogram per microliter level of detection using tandem mass analyzer or high-resolution mass analyzer as detector. If such sensitive and selective mass analyzer can be coupled with GC with spiral LVI, much more sensitivity can be achieved, enabling sub picogram per milliliter level of detection for fluoridated phosphonates from nerve agent adducts.

4. Conclusion

By adopting spiral LVI system, volatile fluoridated phosphonates, ethyl, isopropyl, n-propyl, isobutyl, pinacolyl, cyclohexyl methylphosphonpfluoridates, and *O*-ethyl *N,N*-dimethylphosphoramidofluoridate could be determined with 15–75 pg/mL detection limits and sharp peaks of high number of practical plates. There were considerable variations in the retention times of the target compounds among the experiments, but the retention index confirmation using simultaneous injection of normal alkanes allowed possible identification coupled with confirmation of the tolerance level of the relative peak intensity of identifiable ion to quantitative ion. GC-single quadrupole MS with LVI was applied for the analysis of the target compounds produced by fluoride-mediated regeneration from nerve agent-inhibited plasma. Although matrix components of plasma extract posed interfering effects on sensitive detection, 25–140 pg/mL detection

limits were achieved with sharp peaks. Detection of regenerated fluoridated phosphonates could be achieved for fluoride-mediated regeneration of plasma inhibited by less than 0.3–1 ng/mL levels of nerve agents.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2018.11.011.

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