International Journal of Analytical Mass Spectrometry and Chromatography, 2014, 2, 65-76
Published Online September 2014 in SciRes. http://www.scirp.org/journal/ijamsc
http://dx.doi.org/10.4236/ijamsc.2014.23007

Scientific Research

Improvements to the Fluoride Reactivation Method by Simple Organic Extraction for Retrospective Detection of Exposure to the Organophosphorus Nerve Agents in Human Plasma

Jin Young Lee*, Yong Han Lee

Agency for Defense Development (ADD), Daejeon, Republic of Korea
Email: *marlintime@add.re.kr

Received 3 July 2014; revised 3 August 2014; accepted 2 September 2014

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Abstract

A rapid and simple organic extraction method for the determination of the chemical warfare agent, isopropyl methylphosphonofluoridate (sarin, GB) in human plasma has been developed using gas chromatography-tandem mass spectrometry (GC-MS/MS). In the course of method development, several organic solvents have been screened and chloroform show a low background and increase signal to noise ratio of GB among other organic solvents. Especially, the organic extraction method of reactivated GB from the human plasma has a 30% greater recovery yield than solid-phase extraction (SPE). This simple extraction method was successfully applied to the trace analysis of nerve agents in human plasma in the 3rd Organisation for the Prohibition of Chemical Weapons (OPCW) confidence building exercise on biomedical sample analysis.

Keywords

Chemical Warfare Agents, Isopropyl Methylphosphonofluoridate, Organic Extraction, Plasma

1. Introduction

Chemical warfare nerve agents such as sarin (GB, isopropyl methylphosphonofluoridate) represent some of the most toxic substances available for misuse by nations and terrorist groups. The acute toxicity of nerve agents

*Corresponding author.

How to cite this paper: Lee, J.Y. and Lee, Y.H. (2014) Improvements to the Fluoride Reactivation Method by Simple Organic Extraction for Retrospective Detection of Exposure to the Organophosphorus Nerve Agents in Human Plasma. *International Journal of Analytical Mass Spectrometry and Chromatography*, **2**, 65-76. http://dx.doi.org/10.4236/ijamsc.2014.23007

and organophosphorus pesticides is caused by inhibition of acetylcholinesterase, which produces an excess of the neurotransmitter acetylcholine in central, peripheral, and neuromuscular synapses. The results of excess acetylcholine include miosis, sweating, excessive salivation, muscle fasciculation, seizure, and respiratory failure [1]. Under the Chemical Weapons Convention (CWC) Treaty of 1997, all declared nerve agent stockpiles and chemical weapons production facilities are now scheduled for destruction or inactivation [2]. However, the availability of precursor materials and the relative ease of agent synthesis propagate lingering concern about the use of nerve agents as weapons of mass destruction, even after the destruction of existing stockpiles. Exposure assessment is critical with respect to justifying the course of medical treatment of exposed individuals and preventing further contamination from secondary exposures. Historically, exposure assessment was limited to criteria based on medical observation of the signs and symptoms of cholinergic crisis and blood cholinesterase inhibition. However, cholinesterase inhibition is not specific, and many different chemicals will cause a decrease in enzyme activity. Furthermore, variations in cholinesterase activity within individuals and populations have made minor exposures difficult to detect.

Several analytical methods have been used to monitor nerve agent exposures. Gas chromatography-mass spectrometry (GC-MS) [3], GC-MS/MS [4]-[7], or liquid chromatography-mass spectrometry [8]-[12] methods that measure organophosphorus nerve agents hydrolysis products in urine are sensitive and specific; however, depending on the level of exposure, these metabolites are largely excreted within a few hours, and they are completely excreted within a few days after exposure [13]. Other approaches use the protein adducts formed with nerve agents as exposure markers. One such method involved the pepsin digestion of organophosphorus nerve agents conjugated butyrylcholinesterase (BuChE) from plasma, followed by analysis of the target nonapeptide by LC-MS/MS [14]. This method has the ability to detect nerve agent inhibited BuChE, even after aging has occurred. Another recent analytical development to improve the specificity and sensitivity of exposure assessment includes a GC-MS method for measuring fluoride ion regenerated alkyl methylphosphonofluoridates from plasma butyrylcholinesterase [15] [16]. The advantage of fluoride induced reactivation method is the generation of volatile nerve agents as analytes, which negates the need for derivatization as is necessary with the GC-based alkyl methylphosphonate methods.

In case of the fluoride induced reactivation method, the regenerated nerve agents were enriched by solid-phase extraction (SPE) from plasma and the eluant was analyzed by GC-MS. There are a lot of papers about fluoride reactivation techniques utilizing C18 cartridge for SPE followed by GC analysis [17]-[19]. However, some losses of agents have occurred during the loading and washing step of cartridge in the SPE. Also, the nerve agents could be degraded to alkyl methylphosphonates because water was used in the preconditioning and washing step in the SPE. Thus, this result could cause the loss of recovery of nerve agents.

This paper describes improvements to the fluoride reactivation method by simple organic extraction of GB in plasma followed by analysis using gas chromatography-tandem mass spectrometry (GC-MS/MS). This simple extraction method was successfully applied to the trace analysis of nerve agents in human plasma in the 3rd Organisation for the Prohibition of Chemical Weapons (OPCW) confidence building exercise on biomedical sample analysis.

2. Experimental

2.1. Materials and Reagents

The sarin (GB, isopropyl methylphosphonofluoridate) of purity greater than 90% was synthesized in our laboratory [20] [21]. The purity of synthesized compounds was confirmed by Nuclear Magnetic Resonance (NMR). Analytical grade solvents and reagents were obtained from Aldrich Chemical Company (Seoul, Korea). Centrifugal ultrafilter (Amicon Ultra-2, 3 kDa) was purchased from Millipore (Bedford, MA). Fresh human plasma was obtained from five healthy male Korean volunteers (5 ml per person). High purity water was generated by a Milli-Q filtering system (Millipore, Bedford, MA). All chemicals, solvents, and gases were used as obtained with no further purification.

2.2. Stock Solutions and Calibration Standards Preparation

The stock solution of GB was prepared in 2-propanol at a concentration of 20 μg/ml and stored at −20˚C until used. The working solution (1 μg/ml) was prepared by diluting stock solutions in 2-propanol for optimization of chromatographic and MS conditions. Calibration standards of GB prepared by diluting the stock solution to ob-

tain the following five concentration points: 0.5, 1, 2.5, 5, and 10 ng/ml. All of the calibration standards were also stored at −20˚C until analysis.

2.3. Sample Preparation

2.3.1. Incubation of Plasma Samples with GB

Human plasma which was purchased from Innovative Research (Innovative Research-Novi, Peary Court; Novi, MI, USA) was incubated with GB to inhibit cholinesterase. A solution of plasma (1 ml) in phosphate buffered saline (PBS, 100 µL) was incubated with 20 µL of GB (1 µg/ml, approximately 2.5 equiv) for 3 h at 30˚C in the incubating shaker. After incubation, the sample was transferred to 2.0 ml of the centrifugal ultrafilter (Amicon Ultra-2, 3 kDa) and centrifuged at 13,000 rpm for 10 min to remove the excess of GB. The 0.1 M acetate buffer (0.2 ml) was added to the filter and centrifuged again. This procedure was repeated twice consecutively.

2.3.2. Fluoride-Induced Reactivation of Inhibited Plasma Samples

Human plasma containing GB-inhibited BuChE was diluted with 0.1 M acetate buffer (0.3 ml, pH 4.1), resulting in a reaction mixture with pH 4.5 in the 1 ml vial and incubated with 0.5 M potassium fluoride (0.5 ml) for 30 min at 37˚C in the incubating shaker.

2.3.3. Organic Extraction of Plasma Samples

After reactivation for 30 min, the sample was centrifuged at 13,000 rpm for 5 min and resulting clear supernatant was transferred to 2 ml of microcentrifuge vial. 0.6 ml of chloroform was added and the vial vigorously mixed. The organic layer was transferred to 2 ml tube containing 1 g of anhydrous sodium sulfate. The second extraction with an additional 0.6 ml of chloroform was done and also added to the 2 ml tube. The extract was filtered and concentrated for analysis under a gentle stream of nitrogen at a temperature of 40˚C to a volume of 0.1 ml.

2.4. Instrumentation

2.4.1. GC-MS/MS Conditions

The assay was performed using an Agilent Technologies GC model 7890A (Agilent, USA) interfaced to a tandem-quadrupole MS (Agilent Technologies model 7000 MSD). GC separations were achieved using HP-5MS (Agilent Technologies) column (30 m × 0.25-mm i.d., 0.25-µm film thickness). The carrier gas was helium with a flow rate of 1.2 ml/min. Injection of 1 µL was made by autoinjector (ALS model 7683B, Agilent Technologies) into a splitless injector port at a temperature of 250˚C. The initial oven temperature of 40˚C was held for 1 min, then ramped at 10˚C/min to 100˚C and 50˚C/min to 300˚C held for an additional 5 min.

Samples were ionized by electron ionization with the electron energy of −70 eV in the positive mode. Mass spectra were obtained at a dwell time of 0.1 s for each transition in the multiple reaction monitoring (MRM) mode. Nitrogen was used as the collision gas at a pressure of −2 mTorr with a collision-induced dissociation (CID) energy of 20 eV. The CID energy was optimized for the m/z 99 > 81 transition for GB.

2.4.2. LC-MS/MS Conditions

A Thermo-Scientific Accela LC system was used consisting of a Thermo-Scientific Accela autosampler plus Accela 600 pump. The system was fitted with a 150 mm × 2.1 mm Alltech C_{18} column (Thermo Electron Co., USA), with 5 µm particle size and 100 Å pore size. The mobile phase consisted of water (solvent A) and methanol (solvent B), each modified with 20 mm ammonium formate. The gradient was as follows: 5% of B (from 0 to 2 min), linear increase up to 90% B at 8 min and 100% B at 10 min at a flow-rate of 0.3 ml/min. The injection volume for LC experiment was 10 µL using the autosampler. The column effluent was introduced into a Thermo-Scientific TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Finnigan Surveyor, San Jose, CA, USA) via an atmospheric pressure ionization source/interface operated in eletrospray ionization (ESI) mode. Ionization was performed in negative mode for alkyl methylphosphonic acids. The spray voltage was 2.5 kV, capillary temperature 350˚C, vaporizer temperature 300˚C, sheath gas 40 arbitrary units, and auxiliary gas 10 arbitrary units. Both Q_1 and Q_3 were operated at the peak width of 0.7 amu. The collision gas was argon at 1.3 mTorr. Detection of analytes was conducted in selected reaction monitoring (SRM) mode. The Xcalibur software manufactured by Thermo Scientific was used for instrument control, data acquisition and data handling.

2.5. Quantitation

Calibration standards were prepared from ethyl acetate enriched with concentration of 0.5, 1, 2.5, 5, and 10 ng/ml of native GB. The calibration curve was a plot of the concentrations of the external standards versus the ratio of the areas of the native compound. The concentrations of unknown samples were determined using the slope and intercept calculated by linear regression analysis of the calibration curves.

3. Results and Discussion

3.1. Screening and Optimization of Extraction Solvents

First of all, we tested fluoride reactivation technique using C18 cartridge to investigate loss of GB and degradation of GB to isopropyl methylphosphonate in the loading and washing step of SPE. The degradation product, isopropyl methylphosphonate (IMPA) of GB was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). As shown in **Figure 1**, we confirmed that a 15% of GB against spiked amount of human plasma was eluted in the loading and washing step, and then GB was degraded to isopropyl methylphosphonate (IMPA) because the distilled water was used in the preconditioning and washing step of SPE cartridge (**Figure 1(a)**). The final recovery of GB by eluting ethyl acetate on the C18 cartridge against spiked on the human plasma was 58% yield and confirmed by GC-MS/MS MRM scan mode (**Figure 1(b)**).

To improve the recovery efficiency and reduce the degradation of GB, we studied simple organic solvents extraction method and screened various organic solvents to find optimal extraction efficiency. The extracts from each solvent were analyzed by GC-MS/MS MRM scan mode (m/z 99 > 81). The results were summarized in **Table 1**.

As shown in the **Figure 1**, each solvent showed different retention time of GB according to the solvent polar-

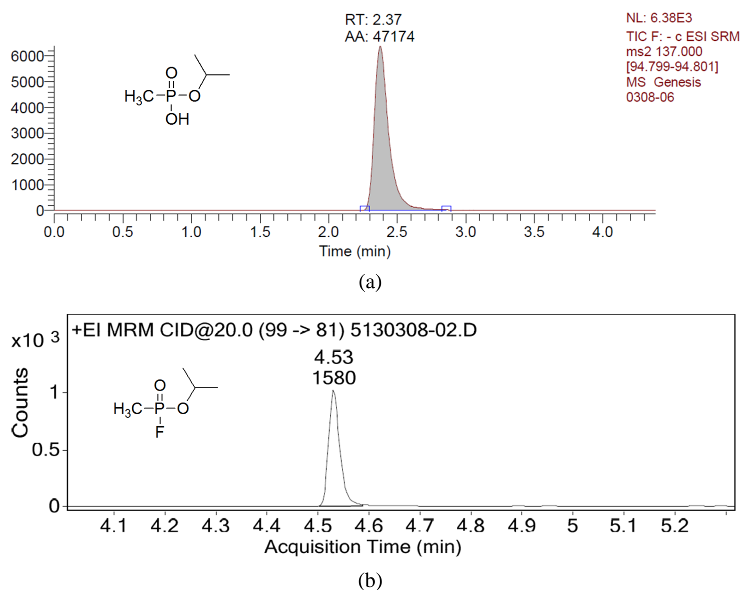


Figure 1. The chromatograms of IMPA and GB in each step, (a) loading and washing, (b) elution.

Table 1. Summary of recovery of GB in each solvent.

No	Solvents	Retention time of GB	Areas of peaks	Recovery yield (%)
1	Ethyl acetate	4.52 min	2497	87
2	Chloroform	4.46 min	2265	79
3	Dichloromethane	4.38 min	1690	59
4	Diethyl ether	4.33 min	1177	41

ity. Compared to the recovery of GB on the SPE, the organic solvent extraction showed generally better recovery yield. Especially, the extraction by using an ethyl acetate showed good peak shape and best recovery efficiency among the tested solvents.

3.2. Precision and Accuracy of Organic Extraction Method Validation

As a part of the validation process, the intraday precision and accuracy of the method were determined by analysing the six replicates on the same day. The precision and accuracy were expressed as relative standard deviation (RSD) and relative error (RE), respectively. The plasma samples were extracted by ethyl acetate and chloroform. The extracts were analysed in six repeated measurements and the intraday precision and accuracy were determined in six replicates. The precision (RSD) for organic extraction of plasma samples, spiked GB was below 1.5%. The accuracy (RE) was in the range from −4.0% to 4.0% for ethyl acetate and −1.4% to 3.7% for chloroform. The accuracy and reproducibility for organic extraction of plasma samples demonstrate the applicability of organic extraction method in a diverse range of biomedical matrices (**Table 2**).

3.3. Optimal Condition for Analysis of Trace Amount of GB in Human Plasma by GC-MS/MS

The ethyl acetate was selected as organic extraction solvent to obtain reactivated GB after reactivation procedure of sarin-inhibited BuChE was done by using 0.5 M potassium fluoride in acetate buffer solution. The analysis of reactivated GB from the human plasma by GC-MS/MS is very challenging because the concentration of GB is low ppb level. To further confirm the structure of GB, the extract was analyzed by GC-MS/MS product ion scan mode. Although an ethyl acetate has good recovery yield, it has a high background with interfering peak at the mass measured for GB. As shown in the GC-MS/MS product ion scan mode (**Figure 2**), the peak of interference was eluted at Rt = 4.50 min adjacent to the peak of GB in ethyl acetate extraction. The GB and interference have two difference product ions, m/z = 81, 47 for the GB and m/z = 71, 43 for the interference from the same quasi-molecular ion, m/z = 99.

When the extraction solvent was substituted for chloroform, the GB eluted at Rt = 4.55 min and the interfering peak was disappeared (**Figure 3**). This result indicated that the chloroform has also a good recovery yield corresponding to that of an ethyl acetate and the substitution of the chloroform for an ethyl acetate as extraction solvent showed a low background and increased signal to noise ratio in the trace analysis of GB from human plasma.

3.4. Application to the Analysis of Unknown Human Plasma Samples Using GC-MS/MS

In the 3rd OPCW confidence building exercise on biomedical sample analysis test scenario, the staff from the facility has been exposed to GB in the synthesis laboratory and blood samples from these four staff were collected, centrifuged into plasma. These four plasma samples (301 - 304) were sent for biomedical sample analysis in support of an investigation of alleged use. There was evidence of GB use and the concentration of the plasma samples was less than 10 ppb. The participating laboratories were asked to identify a GB relevant to the Chemical Weapons Convention (CWC) in the four human plasma samples.

3.4.1. Unknown Human Plasma Samples Preparation

Human plasma samples (301 - 304, 0.5 ml) were transferred to 0.5 ml of centrifugal ultrafilter (Amicon Ul-

Table 2. Interday precision and accuracy of the method (n = 6).

Solvent	No	Extraction yield (%)	RE (%)	RSD (%)	Solvent	No	Extraction yield (%)	RE (%)	RSD (%)
Ethyl acetate	1	90	4.0	0.7	Chlorform	1	80	2.4	1.0
	2	86	−0.5	0.1		2	81	3.7	1.5
	3	83	−4.0	0.7		3	76	−2.7	1.0
	4	88	1.7	0.3		4	77	−1.4	0.6
	5	85	−1.7	0.3		5	77	−1.4	0.6
	6	87	0.5	0.1		6	78	−0.1	0.1

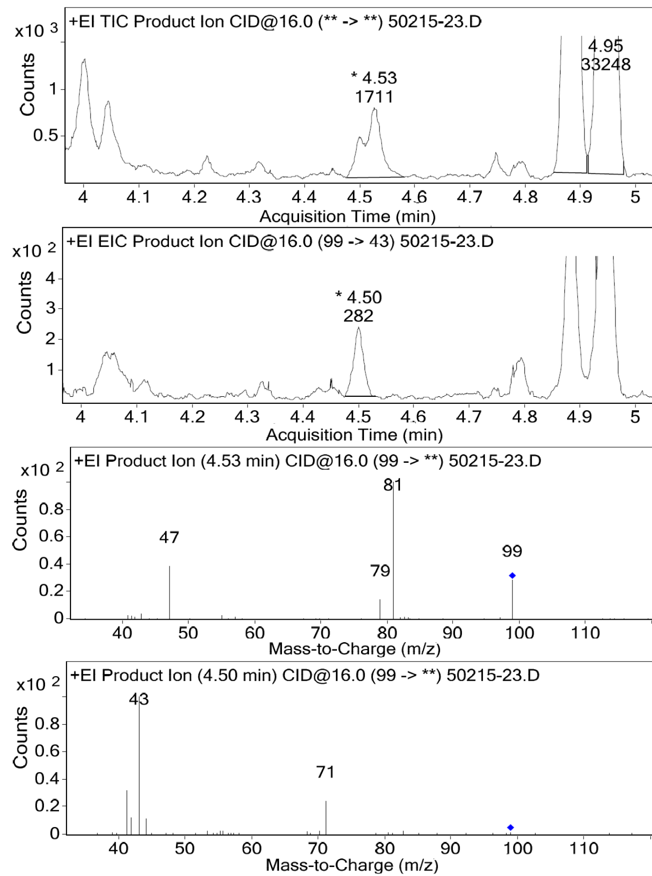


Figure 2. The chromatograms and mass spectra of ethyl acetate extract by GC-MS/MS product ion scan.

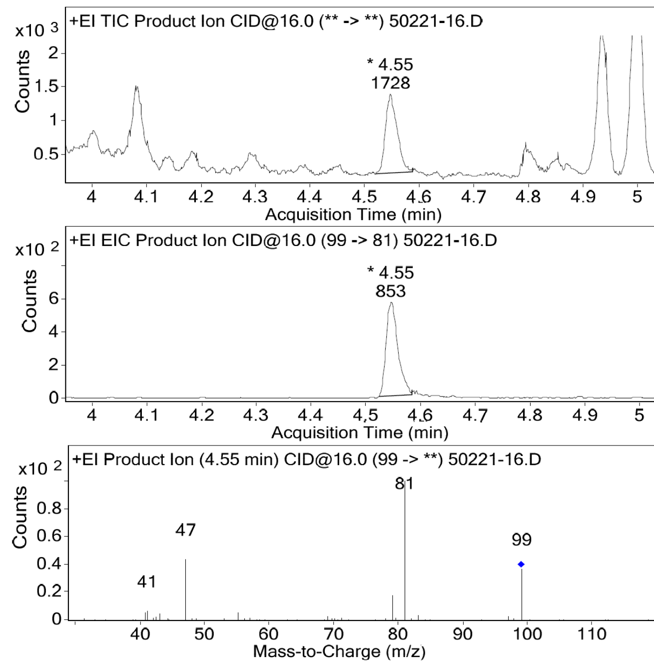


Figure 3. The chromatograms and mass spectra of chloroform extract by GC-MS/MS product ion scan.

tra-0.5, 3 kDa) and centrifuged at 13,000 rpm for 10 min. The 0.1 M acetate buffer (0.2 ml, pH 4.1) was added to the centrifugal ultrafilter and centrifuged again. The supernatant (0.2 ml) was transferred to 1 ml vial and the filter was rinsed with 0.1 M acetate buffer (0.3 ml). The supernatant (0.5 ml) was incubated with 0.5 M potassium fluoride (0.5 ml) for 30 min at 37˚C in the oven. After reactivation for 30 min, the sample was centrifuged at 13,000 rpm for 5 min and resulting clear supernatant was transferred to 2 ml of microcentrifuge vial. 0.6 ml of chloroform was added and the vial vigorously mixed. The organic layer was transferred to 2 ml tube containing 1 g of anhydrous sodium sulfate. The second extraction with an additional 0.6 ml of chloroform was done and also added to the 2 ml tube. The extract was filtered and concentrated for analysis under a gentle stream of nitrogen at a temperature of 40˚C to a volume of 0.02 ml. 2-propanol (0.08 ml) was added to exchange the solvent and concentrated to a final volume of 0.05 ml under N_2 stream for the GC analysis. In the case of 304 plasma sample, the sample was concentrated to a volume of 0.025 ml.

3.4.2. Analysis of Plasma Sample by GC-MS/MS MRM and Product Scan Mode

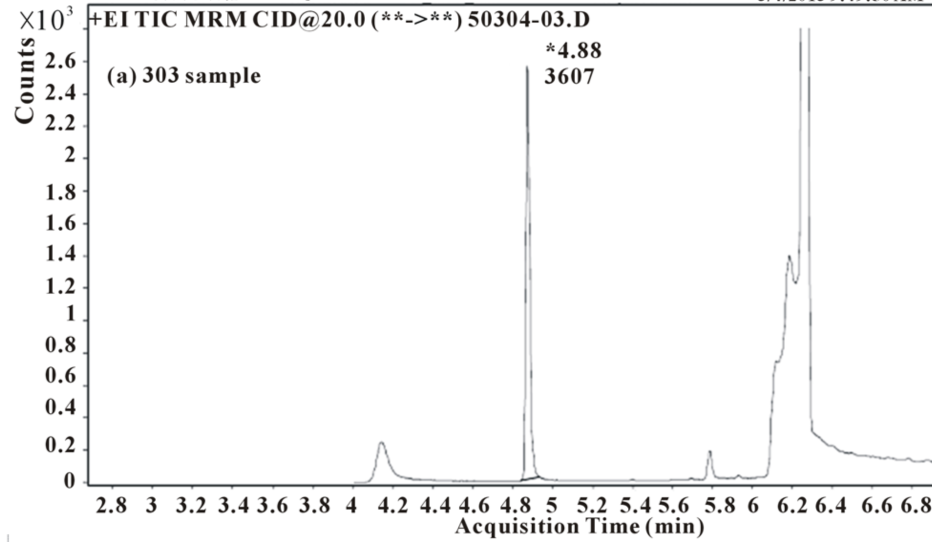
The developed method was applied to the identification for GB present in the unknown plasma samples (301 - 304). To rapidly screen where GB is present in the samples, we selected the common fragment ions at *m/z* 81 and 47 as product ions and analyzed the each plasma sample in the MRM scan mode. As shown in the **Figure 4**, each peak as anticipated for GB was eluted at Rt = 4.88 min and 4.81 min in the 303 and 304 samples in the GC-MS/MS MRM scan mode. Compared to retention time of the peak of standard GB, these two peaks were anticipated as GB due to the same retention time and ion transition (*m/z* 99 > 81, *m/z* 99 > 47). To further confirm these unidentified compounds, the product ion scan analysis was conducted with Q1 set for precursor ion $[M-C_3H_5]^+$ at *m/z* 99 with collision energy 16 eV. The ion at *m/z* 81 was observed as base peak by loss of H_2O from the precursor ion. Also, the ion at *m/z* 47 indicated these unknown compounds have the P=O bond. Based on the result of MRM and product ion scan mode, these two peaks in the plasma samples were confirmed as GB (**Figure 4**).

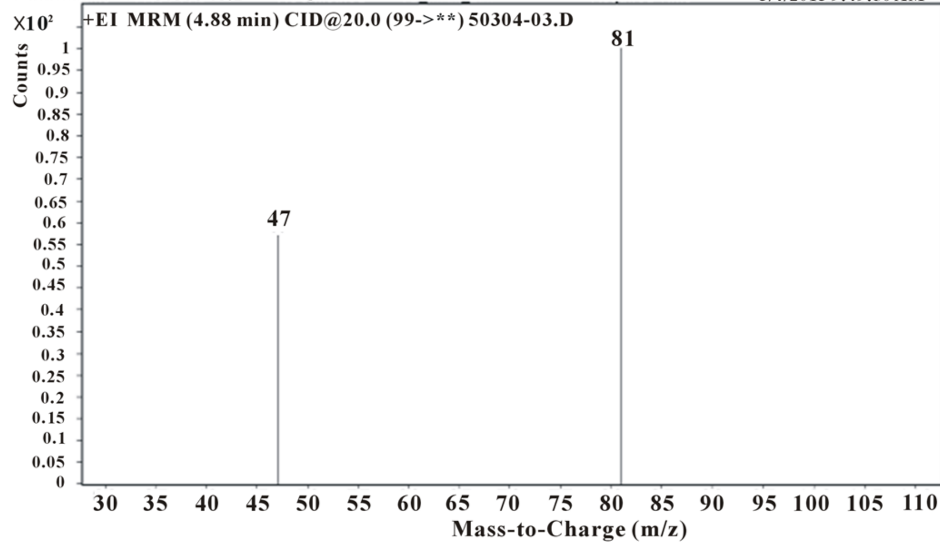
3.4.3. Quantitative Analysis and Limit of Detection (LOD) for Unknown Plasma Sample by GC-MS/MS MRM Mode

To ascertain the reliability of the method, the quantitative analysis of analytes in the unknown samples was carried out at different concentrations. A calibration graph was constructed by plotting the ratio of the peak area for GB within the spiking concentration range of 0.1 - 5 ng/ml in the MRM scan mode. Linearity was observed over the specified concentration range with $\gamma^2 = 0.999$ for GB. The compound which was confirmed as GB in the 303 and 304 plasma samples was analyzed by MRM scan mode to calculate the concentration of a spiked compound. The intergrated area of GB was ratioed against that of the external standard for quantitation purposes. The value of intergrated areas for each analyte is calculated on a calibration curve obtained from the standard samples. In the case of 303 and 304 samples, we reported that GB was spiked at the 2.4 ng/ml for 303 sample and 0.3 ng/ml for 304 sample. However, the real concentration of the spiked chemical in the each sample was 3.0 ng/ml for 303 sample and 0.5 ng/ml for 304 sample. The difference of concentration between reported and spiking was originated from organic extraction yield. Limit of detection (LOD) of GB and quantitative value in the 303 and 304 plasma samples showed in the **Table 3**.

4. Conclusion

The nerve agents are toxic chemical warfare agents, hence their detection and identification are of high importance from verification point of view of CWC. A rapid and simple organic extraction method for the determination of the chemical warfare agent, isopropyl methylphosphonofluoridate (sarin, GB) in human plasma has been developed using gas chromatography-tandem mass spectrometry (GC-MS/MS). In the course of method development, several organic solvents have been screened and chloroform show a low background and increase signal to noise ratio of GB among other organic solvents. Especially, the organic extraction method of reactivated GB from the human plasma has a 30% greater recovery yield than solid-phase extraction (SPE). This simple extraction method was successfully applied to the trace analysis of nerve agents in human plasma in the 3rd Organisation for the Prohibition of Chemical Weapons (OPCW) confidence building exercise on biomedical sample analysis.


Sample Name S303A11
Data Filename 50304-03.D
Position 12
ACQ Method GB_MRM_2.M
Instrument Name 7000 msms
Acquired Time 3/4/2013 9:49:50 AM
Inj VOL 1
+EI TIC MRM CID@20.0 (**->**) 50304-03.D
(a) 303 sample
*4.88
3607
Counts
Acquisition Time (min)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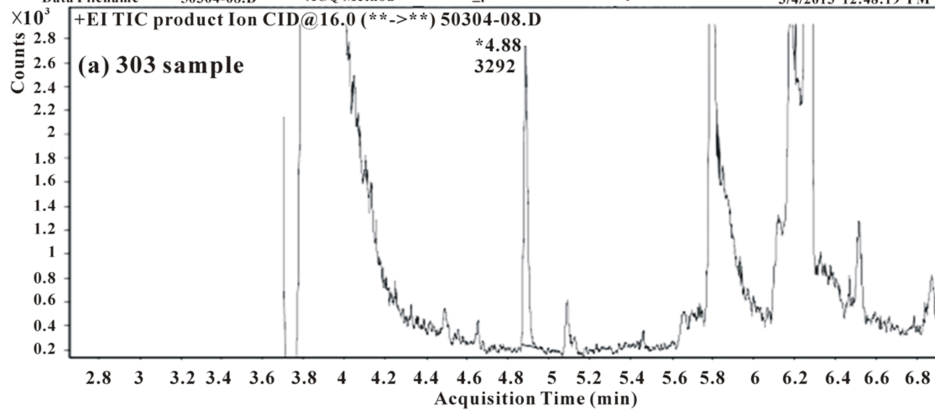
Data Filename 50304-03.D
Position 12
ACQ Method GB_MRM_2.M
Instrument Name 7000 msms
Acquired Time 3/4/2013 9:49:50 AM
Inj VOL 1
+EI MRM (4.88 min) CID@20.0 (99->**) 50304-03.D
81
47
Counts
Mass-to-Charge (m/z)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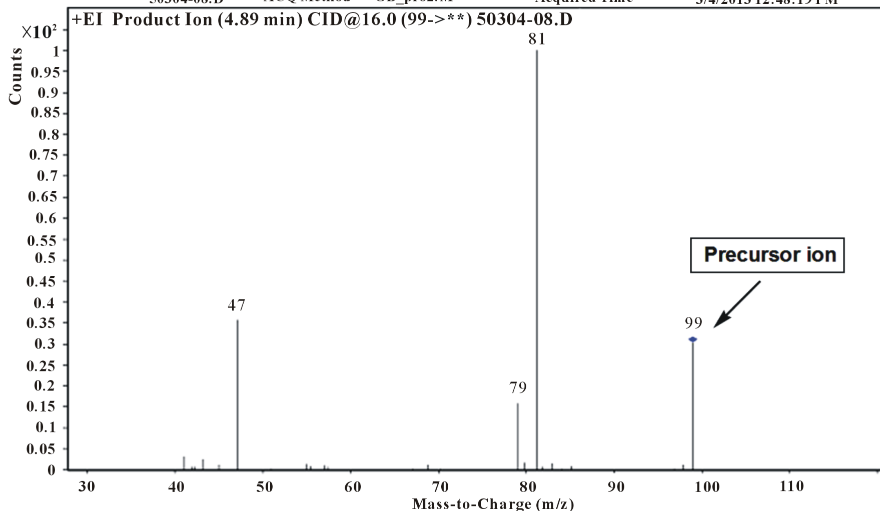
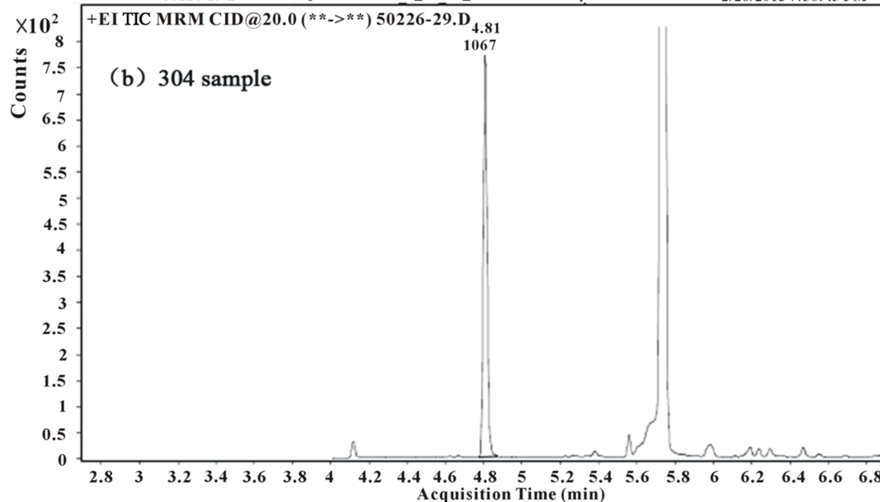
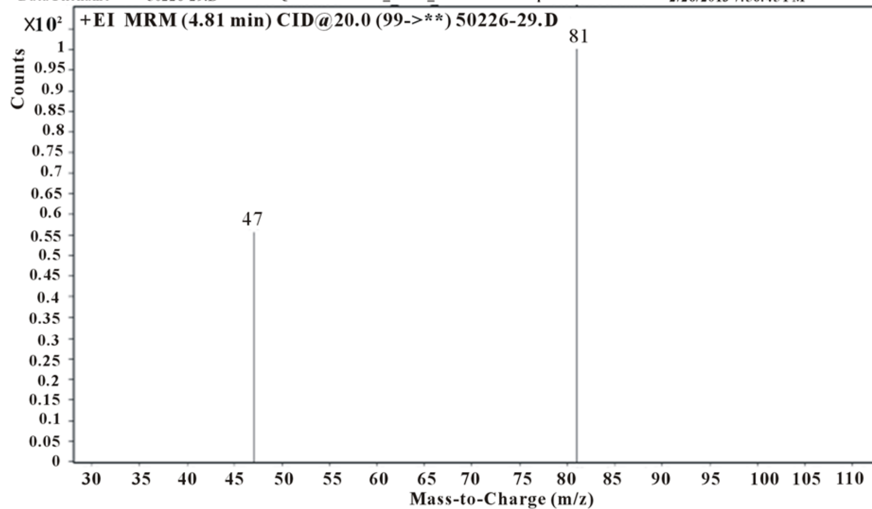
Data Filename 50304-08.D
Position 12
ACQ Method GB_pro 2.M
Instrument Name 7000 msms
Acquired Time 3/4/2013 12:48:19 PM
Inj VOL 1
+EI TIC product Ion CID@16.0 (**->**) 50304-08.D
(a) 303 sample
*4.88
3292
Counts
Acquisition Time (min)


Sample Name S303A11 Position 12 Instrument Name 7000 msms Inj VOL 1
Data Filename 50304-08.D ACQ Method GB_pro2.M Acquired Time 3/4/2013 12:48:19 PM
+EI Product Ion (4.89 min) CID@16.0 (99->**) 50304-08.D
Counts
Mass-to-Charge (m/z)
Precursor ion
47
79
81
99


Sample Name S304A34 Position 12 Instrument Name 7000 msms Inj VOL 1
Data Filename 50226-29.D ACQ Method GB_MRM_2.M Acquired Time 2/26/2013 7:56:45 PM
+EI TIC MRM CID@20.0 (**->**) 50226-29.D
(b) 304 sample
4.81
1067
Counts
Acquisition Time (min)


Sample Name S304A34 Position 12 Instrument Name 7000 msms Inj VOL 1
Data Filename 50226-29.D ACQ Method GB_MRM_2.M Acquired Time 2/26/2013 7:56:45 PM
+EI MRM (4.81 min) CID@20.0 (99->**) 50226-29.D
47
81
Counts
Mass-to-Charge (m/z)

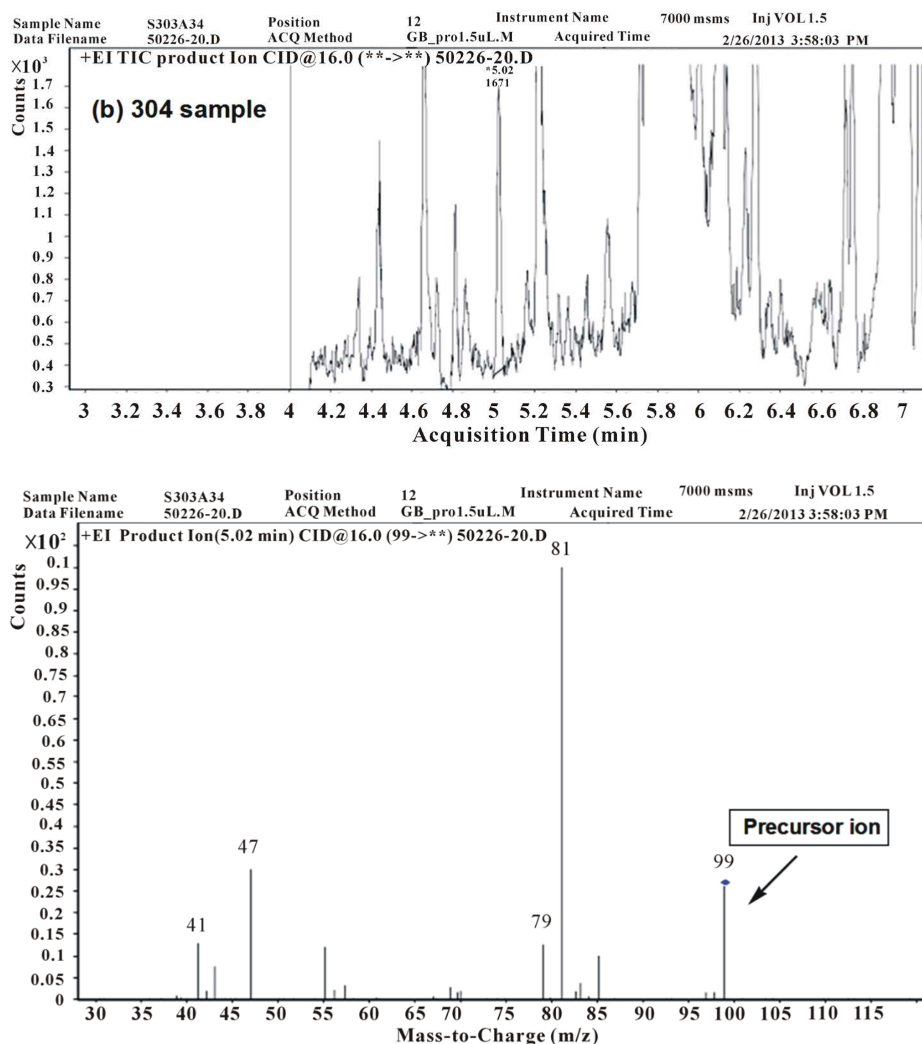


Figure 4. The chromatograms and mass spectra of plasma samples in GC-MS/MS MRM and product ion scan mode, (a) 303, (b) 304.

Table 3. LOD and quantitative data in each plasma sample.

Samples	Chemical	LOD of MRM mode (ng/ml)	LOD of MS/MS mode (ng/ml)	Reported concentration (ng/ml)	Spiking concentration (ng/ml)
301	None				
302	None				
303	GB	0.1	1	2.4	3
304	GB	0.1	1	0.3	0.5

Acknowledgements

We thank the Agency for Defense Development for financial support.

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