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Fluoride reactivation-enabled sensitive quantification of tabun adducts on human serum albumin by GC–MS/MS via isotope dilution

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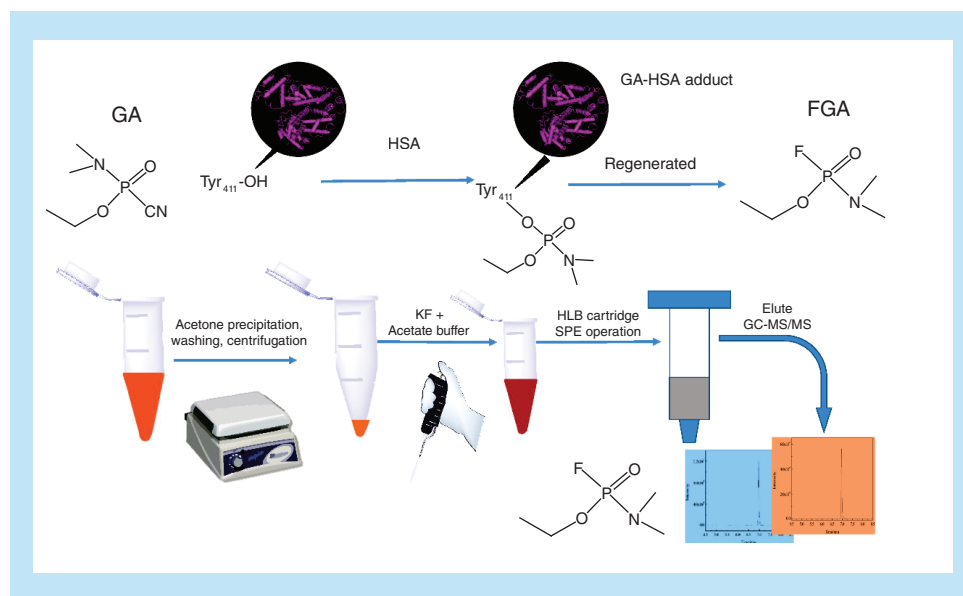
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Organophosphorus nerve agents inhibit the cholinesterase activity by phosphorylation of the active site serine. The resulting phosphorylated cholinesterase and adducts on human serum albumin (HSA) are appropriate biomarkers for nerve agents exposure. Several methods have been developed for the detection of nerve agents, including fluoride reactivation or alkaline cleavage. It was previously thought that some nerve agents adducts to HSA could not be detected via fluoride regeneration. In our study, the results showed that tabun (GA) adducts of HSA could be detected by fluoride regeneration. The sample preparation included acetone precipitation, washing and SPE. Deuterated tabun (d_5 -GA) was applied as the internal standard. The product of regenerated fluorotabun is detected with a good linearity ($R^2 > 0.997$) in the concentration range from 0.02 to 100.0 ng/ml, small relative standard deviation ($\leq 6.89\%$) and favorable recoveries between 94.8 and 106.3%. The established preparation confirmed the fluorotabun was regenerated from the GA-HSA adducts.

Graphical abstract:



Flow diagram for detection and quantitation of tabun adducts to human serum albumin via fluoride reactivation by GC–MS/MS using isotope dilution. HSA: Human serum albumin.

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Keywords: acetone precipitation • fluoride reactivation • GC–MS/MS • HSA adducts • isotope dilution • tabun

Organophosphorus nerve agents (OPNA), such as sarin (GB), soman (GD), tabun (GA) and O-ethyl-S-2-diisopropylaminoethyl methylphosphonothiolate (VX), are the most toxic organic chemicals known as their highly lethal ability [1]. OPNA are easily synthesized [2,3] that have been developed and manufactured since World War II. Even after the ratification by Chemical Weapons Convention (CWC) in Hague in 1997 [4], OPNA still cause critical concern and have already be implemented for several terrorist activities. Large stockpiles of nerve agents might still exist in many countries for possible use or as deterrence. In the Iran–Iraq conflict during the 1980s, nerve agents were thought to be used and the hydrolysis product of GB was found in bomb craters [5]. In an act of terrorism, GB gas was deliberately released in the subways of Tokyo and Masumoto by members of the Japanese cult Aum Shinrikyo in 1995. Thirteen people died, and thousands more required medical care [6,7]. In recent years, there are also still additional attacks using nerve agents, such as the killing of Kim Jong-nam in 2017 and the attacks on Russian spies in 2018. The Organisation for the Prohibition of Chemical Weapons (OPCW) also rebuild the Joint Investigative Mechanism for the use of chemical weapons in Syria.

The CWC went into effect since 1997, and specified the need for the effective extraction and analysis of chemical warfare agents and their environmental markers from environmental samples or biomarkers in the biomedical matrices [8,9]. Therefore, the sensitive detection of OPNA and the related adduct on biomolecules is of significance to confirm chemical attacks. It is reported that the exposure to OPNA rapidly result in the formation of a tight covalent bond between the agent and the serine active sites of cholinesterase. The extreme toxicity of nerve agents results from their high affinity and strong ability to inhibit cholinesterase activity by forming a covalent bond with the serine residue (Ser₂₀₃ for acetylcholinesterase and Ser₁₉₈ for butyrylcholinesterase, respectively) in the active site [10–13]. Also, the nerve agents can form a bond with human serum albumin (HSA; the active site is Tyr₄₁₁) [14–16]. The reaction of OPNA with cholinesterase or HSA results in the loss of a primary leaving groups (fluorine for GB and GD, cyanogroup for GA and a thio-leaving group for VX and RVX, Russian VX) to form a phosphorylated cholinesterase [17]. Thus, these products can act as biomarkers for OPNA poisoning and measurement of these biomarkers in plasma or urine samples can help to identify the actual OPNA [18–21]. Toward this goal, LC–MS/MS and GC–MS/MS have been widely used [22–26]. With the assistance of sample pretreatment including organic solvent precipitation, washing and SPE, the sensitive analysis of OPNA adduct in biological matrix is achieved [27].

To detect OPNA adducts, the generation of OPNA derivate is essential by reactivation techniques [28]. Among them, the fluoride reactivation technique that regenerates phosphyl groups from the cholinesterase upon the incubation with fluoride ions has been widely applied because of the easy operation [29]. Previous study suggested that the products resulting from fluoride ion regeneration were from nerve agent adducts to the cholinesterase, rather than HSA. There was strong evidence that the GB detected in exposed plasma samples was regenerated from BuChE. However, the nerve agents adducts to proteins (e.g., albumin) cannot be reactivated [30,31].

In our study, we employed the pure HSA (commercial) to carry out the fluoride reactivation. The sample preparation method including acetone precipitation, dilution and SPE was optimized to avoid the interference of residual GA. The results in our study confirmed the product based on fluoride regeneration was from the GA–HSA adducts and revealed the applicability of fluoride regeneration for the detection of GA adducts to HSA. The proposed fragmentation pathway of GA was shown in Figure 1. We presented a validated method and new evidence for the sensitive and rapid assessment of GA–HSA adducts via fluoride regeneration.

Experimental

Chemicals & materials

GA, five hydrogen atoms in the ethyl of GA were replaced by deuterium (*d*₅-GA, DGA) and *O*-ethyl *N,N*-dimethylamidophosphofluoridate (fluorotabun, FGA) were synthesized in-house [32], with purity verified at ≥95% by 600 MHz NMR (Bruker BioSpin NMR CP BBO 600S3 [600.13 MHz], with details for GA, FGA and DGA presented in Supplementary Figures 1, 2 & 3, respectively). DGA was stable during the storage and use process in our study. Dichloromethane (LC grade) and water (HPLC grade) were purchased from Honeywell Burdick & Jackson. Acetone (LC grade) was obtained from J&K Scientific. Human albumin was obtained from Sigma-Aldrich at a concentration of 40 mg/ml (the solvent is water). Sodium acetate trihydrate and potassium fluoride were purchased from Aldrich Chemical. Glacial acetic acid was research grade from J&K Scientific. Helium and nitrogen (99.9999% purity) used for GC or sample preparation were purchased from Beijing Beifen Analytical Instrument Company Limited. Oasis HLB cartridges (60 mg/3 ml) and glass autosampler vials (1.5 ml) were

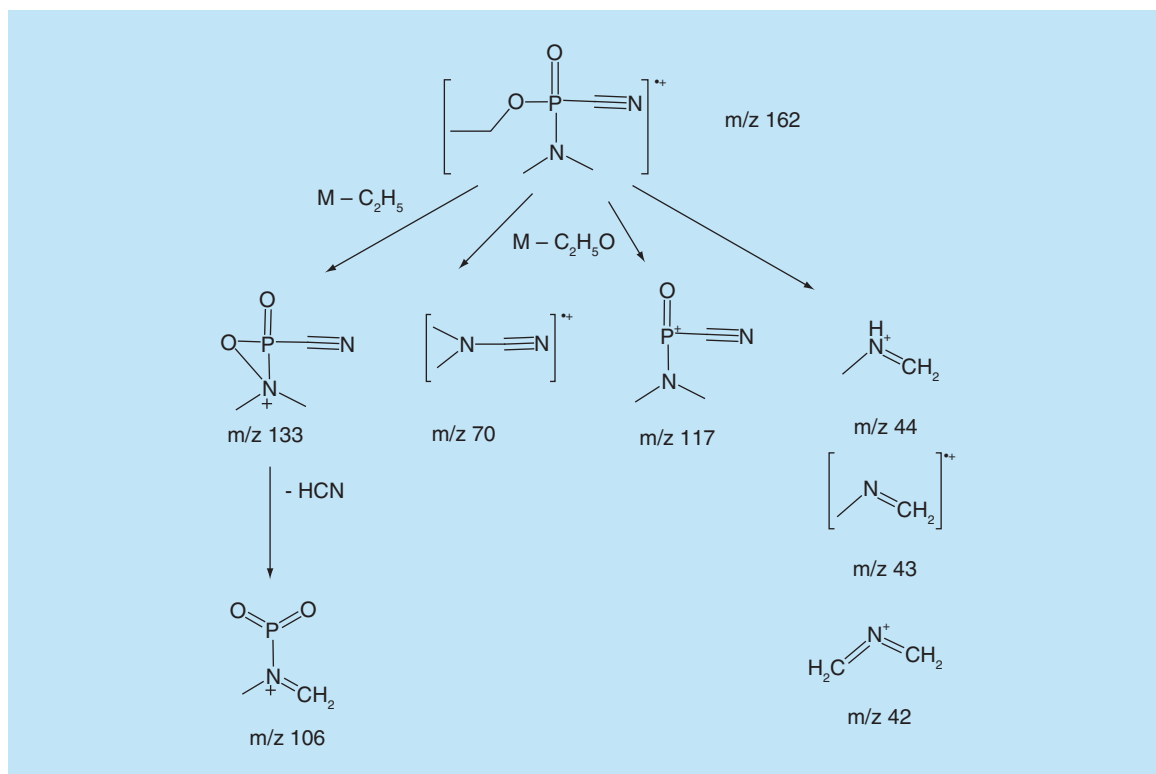


Figure 1. Generalized fragmentation pathway of tabun.

purchased from Agilent (CA, USA). The unknown plasma samples were part of the third biomedical proficiency test scheduled by the OPCW.

Safety consideration

As a dangerous nerve agent and derivatives, GA, DGA and FGA were handled carefully. The preparation of standard solutions and generation of spiked HSA samples were performed in a fume hood by professionals wearing appropriate protective clothing. All equipments that directly contacted GA, DGA or FGA were decontaminated thoroughly with bleach solution.

Standard samples & quality controls

Initial solutions of three analytes (GA, DGA and FGA) were prepared gravimetrically from concentrates in-house and used to make three standard stock solutions at 2.0 mg/ml in isopropanol. One set of GA standard solutions (0.002, 0.010, 0.10, 0.50, 1.00, 5.00 and 10.00 $\mu\text{g/ml}$) was prepared by stepwise dilution of 2.0 mg/ml GA solution into isopropanol to ensure miscibility in HSA. The GA standard solutions (20 μl) were separately added into an HSA sample (1.98 ml, 40 mg/ml) and then incubated for 24 h at room temperature with gentle shaking. The final seven-point calibration curve of GA-spiked HSA ranged from 0.02 to 100.0 ng/ml (0.02, 0.10, 1.0, 5.0, 10.0, 50.0 and 100.0 ng/ml). Similarly, quality control low (QCL, 0.02 ng/ml), quality control medium (QCM, 20 ng/ml) and quality control high (QCH, 80 ng/ml) spiking solutions were prepared by serial dilution of a single high-concentration GA-spiked HSA sample (1.00 $\mu\text{g/ml}$) into nonspiked HSA (both of two concentrations of QCL for 0.02 and 0.06 ng/ml [three-times the LOD] obtained good results and we decided to show the results of the QCL with lower concentration of 0.02 ng/ml). A blank sample was produced using an HSA sample spiked with isopropanol (20 μl) and was used as the matrix for the calibration standards. The other two sets of GA and FGA standard solutions were diluted directly with dichloromethane to establish calibration curves, which ranged from 0.01 to 200.0 ng/ml (0.01, 0.10, 1.0, 10.0, 50.0, 100.0 and 200.0 ng/ml). A working internal standard (ISTD) solution was prepared by diluting DGA with dichloromethane (kept at 4°C until use), and appropriate volumes

Table 1. The optimized selected reaction monitoring transitions parameters for the GC–MS/MS analysis of targets.

| Analytes | Transition ions | Collision energy (V) | Retention times |
|----------|--------------------------|----------------------|-----------------|
| GA | Quantitation transitions | m/z 162→70 | 5 |
| | Confirmation transitions | m/z 133→106 | 8 |
| FGA | Quantitation transitions | m/z 155→127 | 4 |
| | Confirmation transitions | m/z 155→44 | 8 |
| DGA | Quantitation transitions | m/z 167→70 | 5 |
| | Confirmation transitions | m/z 106→43 | 14 |

DGA: Deuterated tabun; FGA: Fluorotabun; GA: Tabun.

were added to a final concentration of 10 ng/ml for each labeled aliquot. All the spiked samples, calibrators, quality control (QC) samples and blank samples were stored at 4°C until they were used.

Sample preparation

Acetone precipitation & washing

A sample set generally consisted of seven calibration standards, the HSA blank and the QC samples. Acetone (2 ml) was added to each sample to precipitate total proteins. The mixed sample was vortexed for 30 s and centrifuged at 5000× *g* for 3 min at room temperature. The supernatant was gently removed and collected without disturbing the protein pellet. The protein pellet was dispersed completely by vortexing and then was resuspended in 2 ml acetone. This operation was repeated three times. Finally, the protein pellet was air-dried in the fuming hood. The supernatants collected for 100.0, 10.0 and 1.0 ng/ml of GA-spiked HSA samples were concentrated with mild nitrogen to 90 µl for GC–MS/MS with 10 µl of 100.0 µg/l DGA as ISTD.

Fluoride reactivation & SPE operation

The process of fluoride reactivation has been widely used for the detection of nerve agents and the conditions had been optimized in other literatures [33]. We also optimized these conditions for the fluoride reactivation. The protein pellets described above were individually dissolved in 1.5 ml of acetate buffer (pH 3.5), followed by addition of 0.2 ml volume of 5.25-M KF solution. The mixed samples were vortexed for 1 min. The appropriate time for incubation was optimized from 20 min to 1 h and we decided 30 min for incubation at room temperature. The samples were centrifuged at 10,000× *g* for 5 min before SPE operation. SPE was performed using Oasis HLB cartridges (60 mg/3 ml), disposable syringes and tubes. The supernatant was loaded onto the cartridge, which was preconditioned with 1 ml methanol and 1 ml water. The cartridge was then washed with 3 ml water and dried with mild nitrogen (drying the SPE cartridge with nitrogen after washing could remove impurities further and totally). The analytes were then eluted with 3 ml dichloromethane. After drying with anhydrous sodium sulfate, the eluent was concentrated with a mild stream of nitrogen gas to 90 µl, and spiked with 10 µl of 100 µg/l ISTD. Each sample was then transferred to the glass insert of an autosampler vial and then analyzed by GC–MS/MS.

Instrumental analysis

The processed samples were analyzed using a Thermo Fisher Scientific Trace GC Ultra with an oil pump and autosampler connected to a Thermo Fisher Scientific TSQ Quantum XLS triple quadrupole mass spectrometer. A DB-17MS/([50%-phenylmethylpolysiloxane], 30 m × 0.25 mm × 0.25 µm) GC column was used and was obtained from Agilent Technologies (CA, USA). The sample injection model was splitless (the time of splitless is 0.7 min) with a glass liner. The temperature of the injector was 250°C. Helium carrier gas was maintained at a constant flow of 1.0 ml/min, and the injection volume was 1 µl. The optimized GC temperature program started at 50°C and was held for 2.0 min, ramped at 10°C/min to 160°C for 0 min, increased to 40°C/min to 280°C and then finally held at 280°C for 3 min to clean the column. The approximate retention times for the analytes were 10.46, 10.39 and 6.97 min for GA, DGA and FGA, respectively.

Mass/mass scan analyses were executed by selected reaction monitoring (SRM). The precursors, the product ions and the collision energies were independently optimized for the three analytes (the data were shown in Table 1, m/z 162→70 and m/z 133→106 for GA, m/z 155→127 and m/z 155→44 for FGA, m/z 167→70 and m/z 106→43 for DGA, respectively). One pair of the precursor and the product ion was monitored for the quantitation and the other was used for confirmation. Ionization mode was by electron ionization using Argon as the collision

gas. The electron energy was set at 70 eV, according to common criteria. The pressure in the collision cell was about 2T. The electron multiplier was optimized at 1580 V. The emission current was 100 μ A. The temperature of the ion source and transmission line were 230 and 280°C, respectively. The rate of the scan was set at 0.05 scans/s and the width of the scan was 0.002 s. First, full-scan program was applied to determine the retention times for GA, DGA and FGA, respectively. Then, we applied the SRM method. The scan time of SRM was divided into three segments for GA (4.5–8.5 min), DGA (4.5–8.5 min) and FGA (8.5–19 min), respectively, which could provide high scan sensitivity.

Data-processing process

The retention times, the integration and selection of peak areas for the analytes were calculated automatically using the Thermo Xcalibur Qual Browser (TXQB) software. The automated data functions as determined by the TXQB software were checked for appropriate smooth points of peak assignment. Quantification of the peaks and the S/N ratio were also calculated with TXQB software, and all samples were processed identically. Individual curves were analyzed using linear regression and were weighted by the reciprocal.

Method validation & calibration curve

The quantitation transition (m/z 155 \rightarrow 127) for FGA and (m/z 167 \rightarrow 70) for ISTD were tested with seven nonspiked HSA samples. Each measurement was performed with a blank sample, three QC samples and a seven-point calibration standard. The LOD and LOQ were evaluated by determination of the S/N ratio (≥ 3 for LOD and ≥ 10 for LOQ, respectively) to confirm and quantitate FGA. The intraday precision and accuracy were calculated with three calibrators (0.05, 5.0 and 100.0 ng/ml) and three QC samples (QCL, QCM and QCH) freshly prepared and analyzed independently ($n = 5$) the same day of use. At least ten measurements were completed within 2 weeks by two analysts, and the resulting data were used to calculate the linearity, the interday precision and the accuracy of the method.

Recovery of sample preparation

Recovery for the method was determined by measurement of spiking samples after whole-sample preparation. All the calibration samples (0.02–100.0 ng/ml) were processed as described above to compare recoveries at various levels and then were subjected to GC–MS/MS analysis. Theoretical molar concentration for regeneration of FGA (TC) can be calculated based on the molar ratio of GA and FGA was 1:1 of the reaction. The actual molar concentration for FGA (FC) after sample preparation was calculated by comparison the peak area of the product to the established calibration curve of FGA. Recovery of reaction was characterized as $(FC/TC) \times 100\%$.

Results & discussion

Use of DGA as ISTD

Deuterated compounds have been widely used as ISTD in numerous methods for the retrospective quantification and detection of chemical agent exposure [34]. In our research, DGA was used as the ISTD to quantify GA and FGA. The standard curves of GA and FGA were established and the results are shown in Table 1. The purpose of GA standard curve is to detect the residual of GA after the operation of ‘acetone precipitation and washing’.

In our study, we applied the process of acetone precipitation and washing to avoid the interference of residual GA. The supernatants after every time of acetone precipitation and washing were collected for 100.0, 10.0 and 1.0 ng/ml of GA-spiked HSA samples. If the ISTD was added prior to the process, the amount of the ISTD could not be consistent after every time of washing, which might influence the sensitivity and stability of the instrument analyze and the standard curve. So, we decided to add the ISTD into the final prepared sample to obtain higher sensitivity and avoided loss during sample preparation.

GC–MS/MS method optimization

In previous studies, the quantification and confirmation of specific ions were optimized. In this study, GC–MS/MS was used for detection. The fragmentation of GA, FGA and DGA resulted in predominant products ions at m/z 163, 133, 106, 70; m/z 155, 127, 44; and m/z 106, 43, 70, respectively. The chemical structures and ions mass spectra of GA, FGA and DGA were shown in Figure 2A–C. All quantification and confirmation transitions were formed by electron ionization fragmentation in the source. Spectra were collected at an electron energy of 70 eV, with the mass range of m/z 33–550. The precursor and product ions spectra for GA, FGA and DGA were

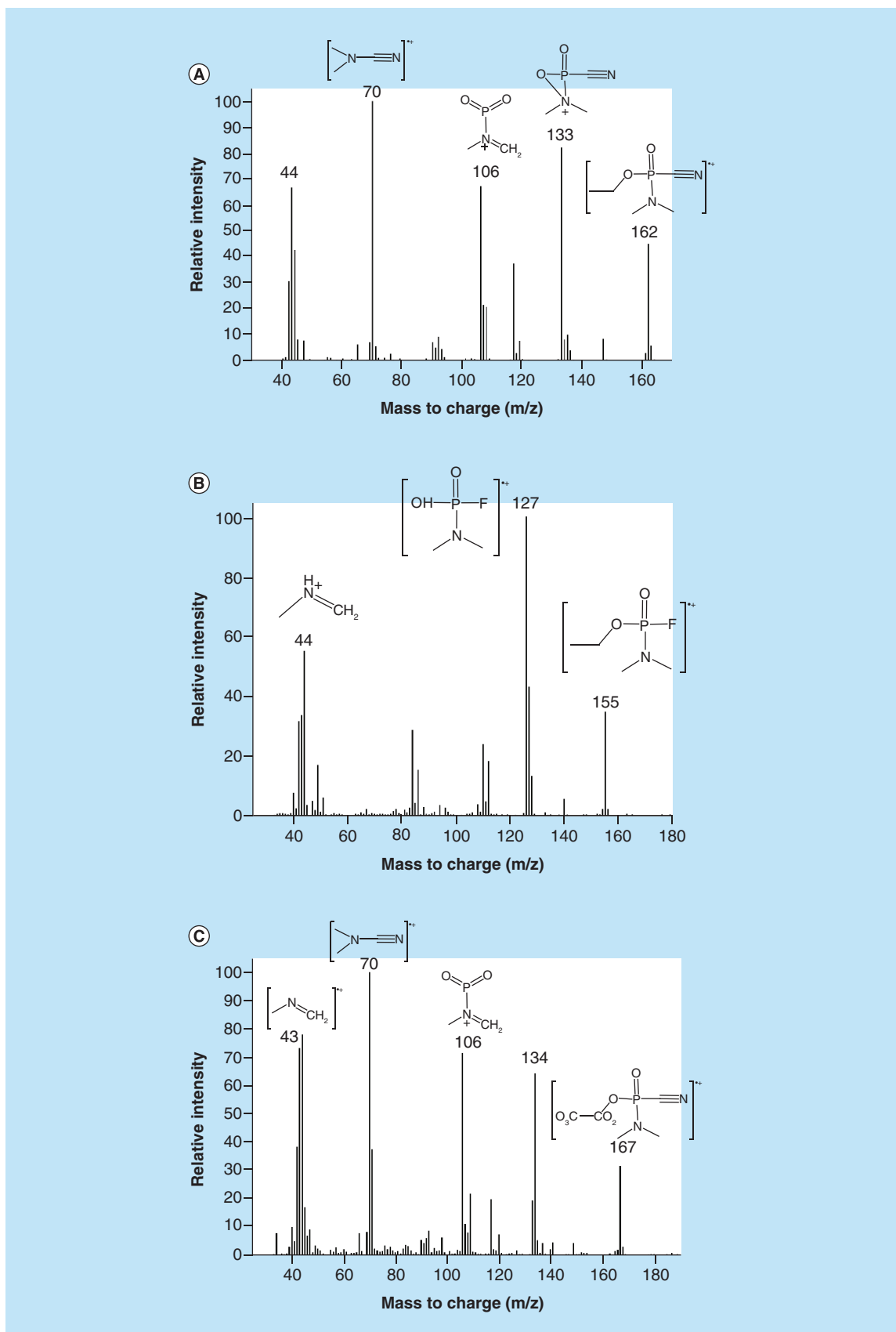


Figure 2. Mass spectrum for the GA, FGA and DGA. Chemical structures and product ion mass spectra of the precursor ions of (A) GA (m/z 162), (B) FGA (m/z 155) and (C) DGA (m/z 167). Spectra were collected at electron energy of 70 eV over the mass range m/z 33–550. Those fragment ions monitored by SRM are labeled with m/z values in structures.

DGA: Deuterated tabun; FGA: Fluorotabun; GA: Tabun; SRM: Selected reaction monitoring.

investigated, and the collision energy of transitions for GA, FGA and DGA was optimized using Thermo Xcalibur Qual Browser software (Thermo Fisher Scientific). Table 1 shows the quantification and confirmation transitions for the targets, acquired at unit resolution, and the data in Figure 2A–C showed the similar MS fragmentation behaviors. Also, GC conditions were optimized for good separation, reducing noise levels as well as a significantly reduced run time (~19 min per sample) to detect FGA.

Mass spectrometric fragmentation patterns

The quantification transitions of m/z 167→70 for DGA corresponded with m/z 162→70 for GA, and represented the structure of the two $C_3H_6N_2^+$ due to the recombination of $-C_2H_6N$ and $-CN$ that were both cleaved from the molecular ion of GA (m/z 162, native). The confirmation for GA was based on the transition m/z 133→106, representing the loss of the $-HCN$ from $C_3H_6PO_2N_2^+$ (m/z 133), which was due to the cleavage of $-C_2H_5$ from the molecular ions of GA. Quantification for FGA was based on the transition m/z 155→127, representing the cleavage of $-C_2H_4$ for neutral loss from the molecular ion of FGA (m/z 155, native). The confirmation for FGA was based on the transition m/z 155→44, representing the cleavage of group of dimethylamino ($C_2H_6N^+$). Also, GC conditions were optimized for good separation, reducing noise levels as well as a significantly reduced run time (~19 min per sample) to detect FGA via fluoride reactivation from the HSA.

Optimization of precipitation of HSA & washing process for the residual GA

Fluoride reactivation is effective and highly sensitive for detection of GA adducts in plasma because GA can be directly changed to FGA by fluoride reactivation in a water matrix. For the real samples, the purpose of detection is to prove the existence of nerve agents (such as GA or GB) in the biological matrix, the residual GA in the biological matrix is not an influence factor. However, our purpose for this study is to reveal the applicability of fluoride ion regeneration for the detection of GA adducts to HSA. We must avoid impurities and interference to be sure that the product of FGA is due to reactivation from GA–HSA adducts, rather than residual GA in water.

In our method, acetone precipitation and washing, which was of great significance for our sample preparation, were introduced to remove all potential GA residues in the matrix. Only after the process of protein precipitation and protein washing, we could confirm that there is no residual GA in the matrix. We collected the supernatant after every time of ‘acetone precipitation and washing’ for the QC samples (QCL, QCM and QCH) and detected the residual of GA by the standard curve of GA. The results indicated that three repeats of acetone precipitation and washing were sufficient for removing all of the GA residues for the samples spiked with different concentrations of GA. The remaining GA in the matrix for the 20 ng/ml GA–HSA adducts was measured after three rounds of acetone precipitation and washing, as shown in Figure 3. Obviously, there was no residual GA. Our use of acetone precipitation and cleaning to remove any residual GA in the matrix allowed confirmation of albumin as the only source of reactivated agent [30].

Optimization of cartridges, eluting solvents, & washing & eluting volumes

The system of fluoride reactivation has been extensively investigated in recent decades and the conditions have been optimized by many reports [34]. Here, only the conditions of SPE in our study were optimized for good separation and recovery of product from GA–HSA adducts by fluoride reactivation. The calibration curve of FGA was established using DGA as ISTD. FGA (1.0 µg/ml) was added into water and the peak areas were measured after SPE operation. The optimum conditions were established by analysis of three factors (cartridges, eluting solvents and the volume of washing and eluting) and three level orthogonal experiments. HLB, C18 and Nexus cartridges, the capacity for all of which were 60 mg/3 ml, were evaluated for recovery. The tested eluents were dichloromethane, acetonitrile and ethyl acetate. The washing and eluting volumes tested were 1, 2 and 3 ml. The orthogonal test results indicated significant effects of different cartridges and eluents. The HLB cartridge provided much higher recovery, which might reflect the hydrophilic–lipophilic balance of the polymeric sorbent material. Dichloromethane was selected as the SPE eluent, with high eluotropic strength and the most chromatographically compatible of the tested eluents. The 2 and 3 ml volumes for washing and eluting showed little difference, but both performed better than 1 ml. Above all, HLB cartridge (60 mg/3 ml) was selected with the dichloromethane as eluent for the SPE process, using 3-ml volumes for both washing and eluting.

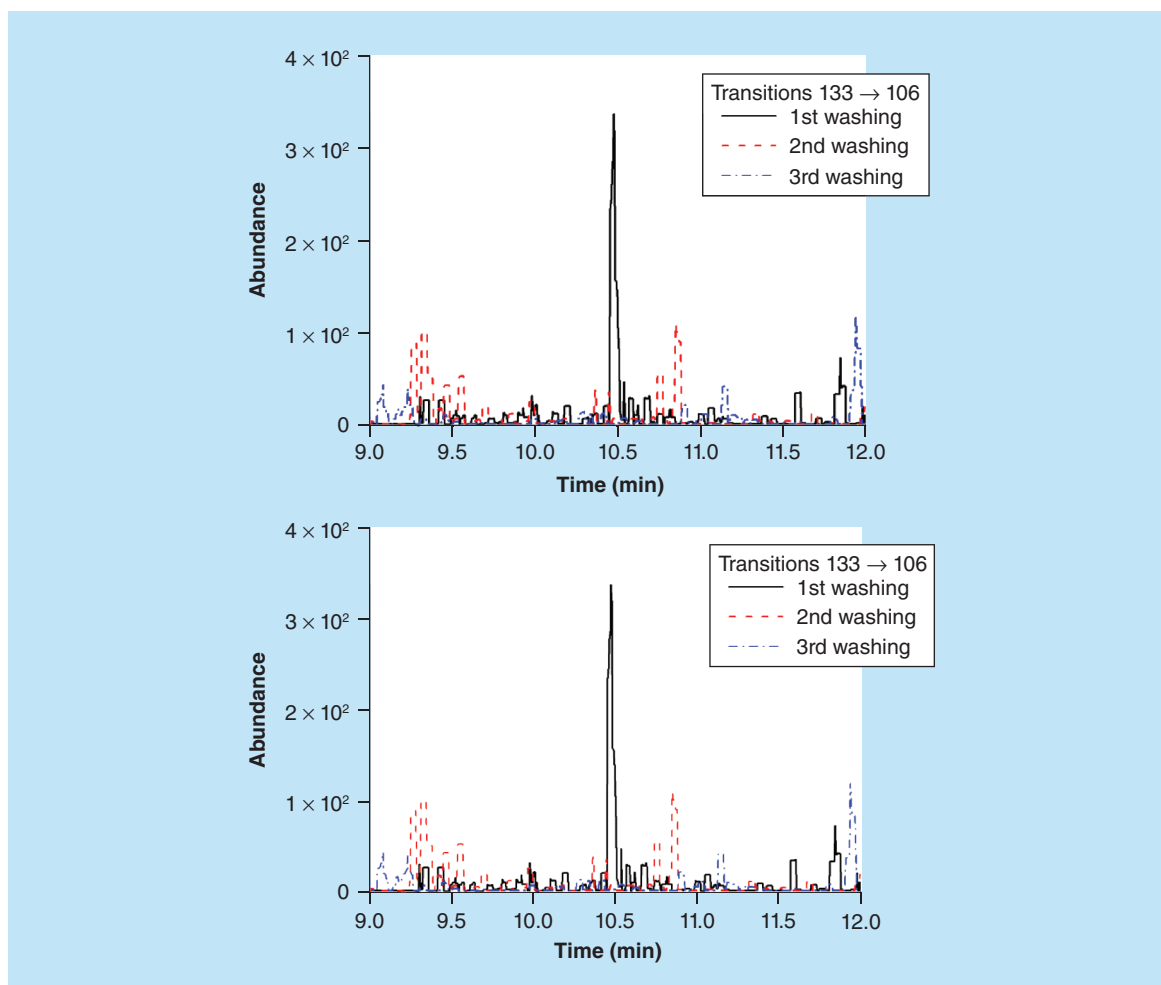


Figure 3. GC-MS/MS selected reaction monitoring chromatogram comparison of the residue of tabun (in the 20 ng/ml tabun-human serum albumin adducts) in the matrix after acetone precipitation and three washing steps. The sample after the first washing is indicated by the black line, and the samples after the second and third washing are indicated by red and blue lines, respectively.

Specificity

The specificity was estimated by measuring six individual HSA samples unexposed to GA. The specificity of the method usually focus on the confirmation of the target object. So, the confirmation transitions were applied to estimate the specificity. No background/interference peaks were present both at the confirmation transitions for m/z 155 \rightarrow 44 of FGA or m/z 106 \rightarrow 43 of ISTD within ± 2.0 min of the corresponding retention time, suggesting good specificity for the instrument method. Also, no significant background/interference peaks (S/N-3) for different individuals were found within ± 2.0 min of the corresponding retention time for transition m/z 155 \rightarrow 44 of the analyte and transition m/z 106 \rightarrow 43 of ISTD.

Linearity & LOD

The peak area ratios of FGA via GA-spiked concentrations in HSA samples exhibited excellent linear relationship in the range from 0.02 to 100.0 ng/ml (shown in Figures 3 & 4). The average calibration equation was calculated as ($y = 0.1966x - 0.094$) with a coefficient of $R^2 = 0.9972$. When the concentration increased beyond 100.0 ng/ml, the upper limit of quantification, there was a GA dose-dependent increase of the peak areas with lower effect than expected from linear regression. The LOD was estimated as 0.02 ng/ml (S/N-3) based on the S/N ratio estimation method. The LOD peak was clearly distinguished as that of the matrix blank sample, as illustrated in Figure 5. The

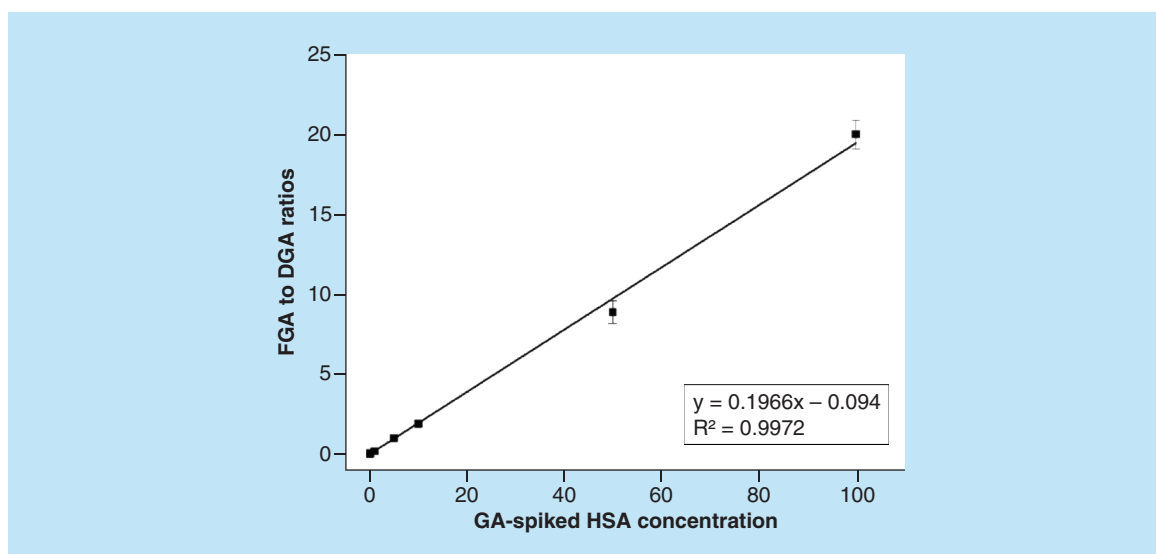


Figure 4. Comparison of tabun-spiked human serum albumin concentration using fluorotabun to deuterated tabun ratios to tabun-spiked concentration.

Table 2. The standard curve of tabun and fluorotabun with the calculated concentration, accuracy and precision of quality control samples for the detection of fluorotabun.

| Analytes | | Linear range | | Linear equation | | R ² | |
|-----------|-----------------------------------|----------------------------------|--------------|----------------------|----------------------------------|----------------|---------|
| GA | | 0.01–200 ng/ml | | y = 0.3227x - 1.6414 | | 0.9985 | |
| FGA | | 0.01–200 ng/ml | | y = 3.4355x + 6.0274 | | 0.9984 | |
| Sample ID | Theoretical concentration (ng/ml) | Intraday (n = 5) | | | Calculated concentration (ng/ml) | Accuracy (%) | RSD (%) |
| | | Calculated concentration (ng/ml) | Accuracy (%) | RSD (%) | | | |
| QCL | 0.02 | 0.02 ± 0.003 | 105 | 5.64 | 0.02 ± 0.004 | 106.3 | 5.88 |
| QCM | 20.0 | 19.0 ± 1.8 | 94.8 | 3.52 | 19.4 ± 1.4 | 97.2 | 4.64 |
| QCH | 80.0 | 81.8 ± 2.1 | 102.3 | 5.22 | 84.3 ± 2.9 | 105.4 | 6.89 |

FGA: Fluorotabun; GA: Tabun; QCH: Quality control high; QCL: Quality control low; QCM: Quality control medium; RSD: Relative standard deviation.

LOD is nearly close to 5.5–16.5 pg/ml for the study using modified methods for the detection of OPNA–protein adducts via refluoridation [30].

Accuracy & precision (relative standard deviation)

The intraday and interday accuracy and precision were measured for three levels of QC samples for the analyte. The intraday accuracy ranged between 94.8 and 105% with precision of ≤5.64% relative standard deviation (RSD; n = 5; Table 2). The interday accuracies were between 97.2 and 106.3% with precision of ≤6.89% RSD (n = 10) for the last 2 weeks of operation (Table 2).

Recovery of reaction & stability

The recovery of the reaction was characterized by calculation of the theoretical molar concentration for FGA based on spiking-GA (TC) by fluoride ion regeneration (molar ratio for GA and FGA was 1:1). The generated molar concentration for FGA was calculated by the established standard curve of FGA (n = 5). The recovery was 36.2 ± 6.57% in QCL, 41.1 ± 5.82% in QCM and 48.2 ± 3.63% in QCH HSA samples. The recovery of QCH was 12 points higher than QCL due to the loss of the analytes in the sample preparation was more significant at low concentration. The results indicated stable reaction recovery across samples with GA-spiked concentration

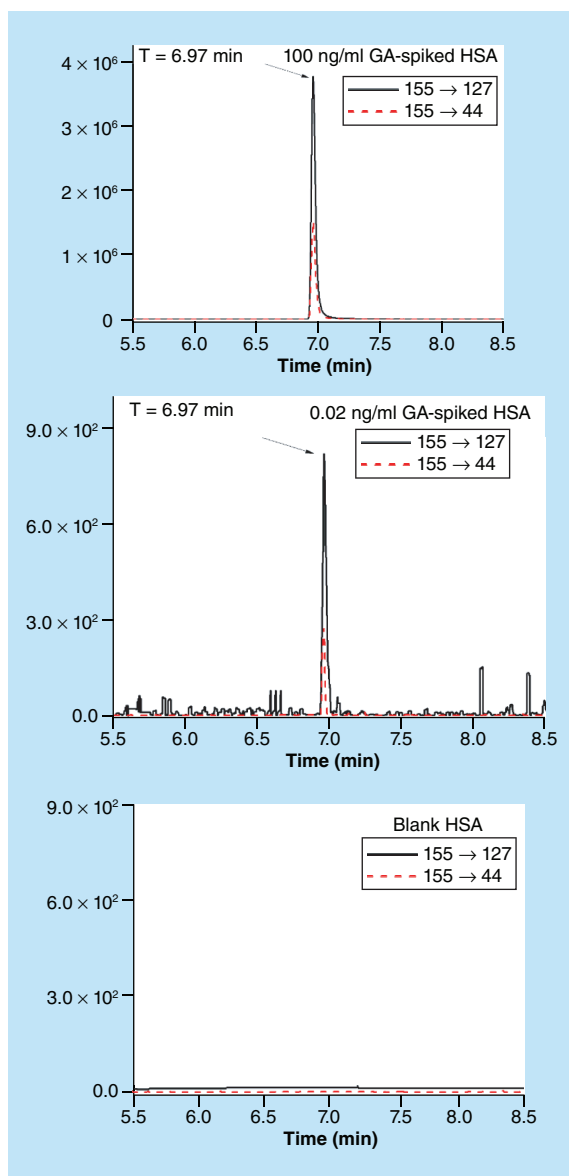


Figure 5. GC-MS/MS selected reaction monitoring chromatogram comparison of 100.0 and 0.02 ng/ml tabun-spiked human serum albumin samples and blank samples.

from QCL to QCH. The stability of the analyte in the processed samples was evaluated at three QC levels (QCL, QCM and QCH) with triplicates. The analytes in all QC samples were stable within $\pm 12.5\%$ ($n = 10$) at different temperatures (4°C , room temperature -25°C and body temperature for 37°C) for 2 weeks.

Application in the biomedical proficiency testing

In the OPCW's 3rd Biomedical Proficiency Testing, six plasma samples (signed 301–306) were collected from residents after an airstrike on a territory under conflict in a residential area. The patients experienced shortness of breath, disorientation, nausea, vomiting and even loss of consciousness (according to the test plan and the scenario given by OPCW). The first responders suspected a nerve agent attack, and the concentration of the nerve agent in the plasma samples was measured at a low to 1 ng/ml level (the nerve agent plasma concentration on-site was usually indicated by the inhibition rate of cholinesterase measured by the Cholinesterase Test Kit). The participating laboratories were asked to test the plasma samples for the presence or absence of biomarkers of nerve agent exposure.

Sample preparation

Because the matrix of the samples was plasma and the purpose of Biomedical Proficiency Testing was to scan and identified the nerve agent in the sample, we compared the sample preparation with and without pre-processing of

acetone precipitation. For the samples without pretreatment, 0.5 ml of plasma sample was mixed with 1.5 ml of acetate buffer (pH 3.5) and 0.2-ml volume of 5.25-M KF solution. The mixed samples were vortexed for 1 min and incubated for 30 min at 25°C. The samples were centrifuged at $13,000 \times g$ for 5 min. Then, the supernatant was loaded onto Oasis HLB (60 mg/ 3 ml) preconditioned with 1 ml methanol and 1 ml water. The cartridge was then washed with 3 ml water and dried with mild nitrogen. The analytes were then eluted with 3 ml dichloromethane. After drying with anhydrous sodium sulfate, the eluent was concentrated with a mild stream of nitrogen gas to 100 μ l and then analyzed by GC-MS/MS. For the plasma with acetone precipitation, the sample preparation was the same to the sample preparation of HSA. A set of plasma samples collected from our lab staff served as blank samples and a 4 ng/ml GA-spiked plasma sample was used as the control.

Analysis of plasma samples by GC-MS/MS

We applied our methods to identify the biomarkers of nerve agent exposure in the six plasma samples. To rapidly screen GA in samples, we selected two transitions: m/z 155 \rightarrow 44 and m/z 155 \rightarrow 127 (m/z 155 \rightarrow 44 and m/z 155 \rightarrow 127 were used both for identify and method validation of FGA as the product of fluoride ion regeneration of GA-HSA adducts). The remaining instrument parameters were the same as described in previous experiments. The ratios of the confirmation transitions to the quantitation transitions for the samples were also recorded by calculated the peak for the two transitions. The ratios of m/z 155 \rightarrow 44 (confirmation transitions) to m/z 155 \rightarrow 127 (quantitation transitions) for the spiked sample (302, 303, 304 and 305) were stable within $38.0 \pm 2.0\%$. The ratio identified that the peak in the chromatograms of sample (302–305) represented GA and showed the chemical purity about the experiment. The results were shown in Figure 6. Using the 4 ng/ml GA-spiked plasma as the control samples, the peak areas of the samples preprocessed by acetone precipitation and washing were close to those observed for samples prepared via direct fluoride reactivation (85.2–90.5%). GA was identified in plasma samples 302, 303, 304 and 305, analyzed by SRM scan mode with samples 301 and 306 used as blank samples. The results indicated that the method we established in our study could also be used in the Biomedical Proficiency Testing.

Conclusion

The aim of our research was to reveal the applicability of fluoride ion regeneration for the detection of GA adducts to HSA. We presented a validated method and new evidence for the sensitive and rapid assessment of GA-HSA adducts via fluoride regeneration. HSA spiked with GA was precipitated and the protein pellet was washed with acetone three-times. The washed pellet was then dissolved in acetate buffer for fluoride reactivation. The mixture was then subjected to SPE and the eluent analyzed by GC-MS/MS with DGA as the isotopically labeled ISTD. The linear range of the method was from 0.02 to 100.0 ng/ml GA exposure ($R^2 > 0.997$) with precision of $\leq 6.89\%$ for RSD and accuracy that ranged between 94.8 and 106.3%. The established preparation method successfully avoided interference of residual GA in water matrix, which means that the detected FGA was regenerated only from the GA-HSA adducts. It is confirmed that the reactivation of GA adducts on HSA could be realized by fluoride reactivation technique for the first time. To confirm the ability of labs to meet treaty compliance requirements and investigate potential events, the OPCW has established a proficiency-testing program that includes an analytical system comprised of 17 expert-designated laboratories. The method we established for GA detection was successfully used in the 3rd Official OPCW Biomedical Proficiency Test [35]. The results were confirmed as completely accurate by OPCW. This study provides a practical method for the analysis of GA-spiked plasma/HSA samples and a new strategy to verify CWC in biomedical samples. The results may indicated that different intoxication mechanisms for GA compared with other OPNAs, which might be due to the difference of -CN and -F from GA and GB, respectively.

Future perspective

The research about biomarkers for OPNA identification and measurement of these biomarkers in biological samples has attracted more and more researchers' attention in the world. Many biomarkers have been selected and obtained by LC/Q-TOF, LC/MALDI-TOF and so on, but the investigations for the mechanism of adducts about OPNA and plasma still remain difficult. The ratio for adducts of OPNA-cholinesterase to that of OPNA-HSA in the plasma needs to be pay more concerns. Also the different nerve agents, such as GA and GB, may show different abilities to form the covalent bond with cholinesterase or HSA in the plasma. In our research, we successfully confirmed the process of fluoride ion regeneration in the detection of GA in the spiked HSA samples. The research in the

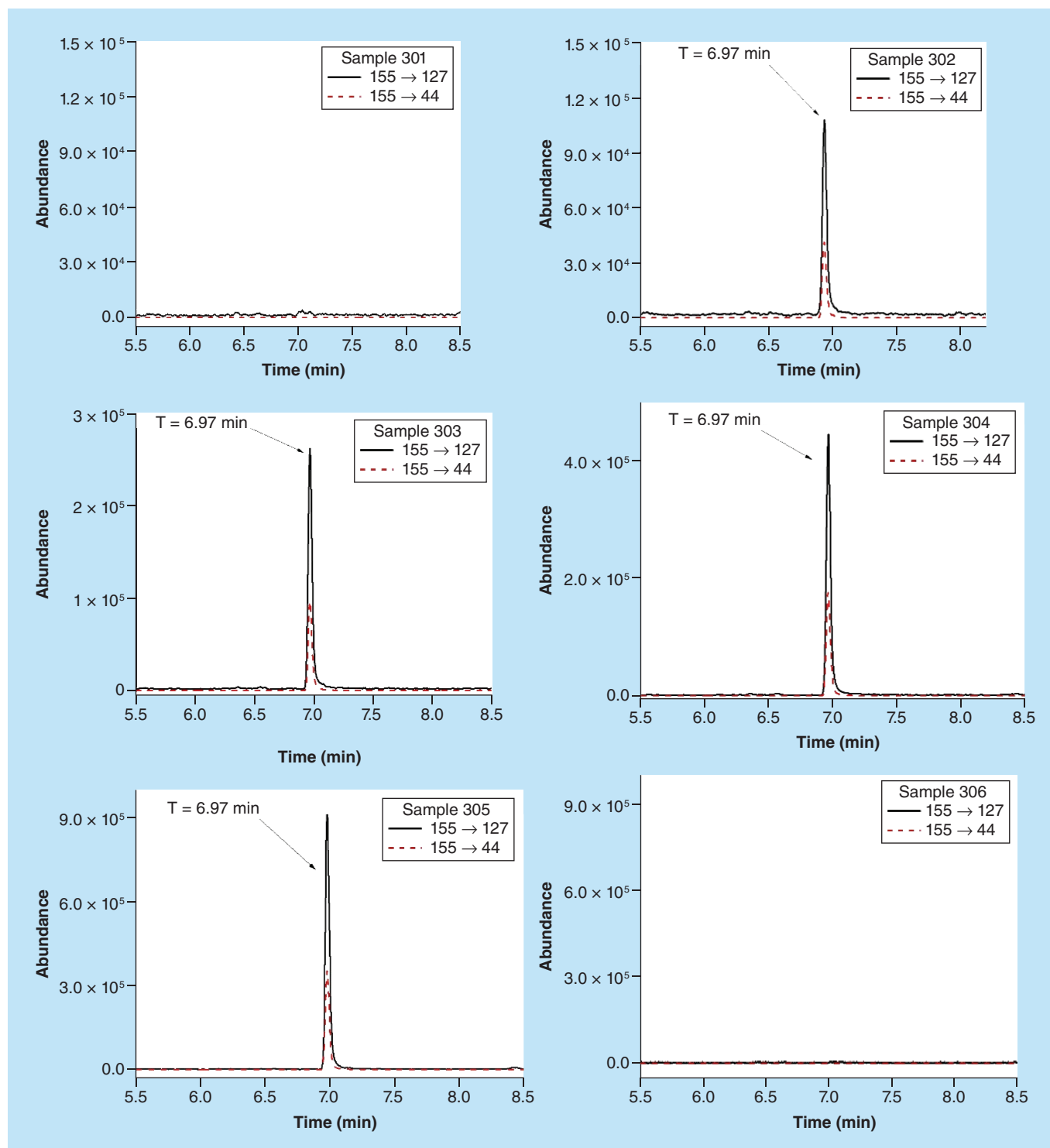


Figure 6. GC-MS/MS selected reaction monitoring chromatogram of samples 301–306 in the Organisation for the Prohibition of Chemical Weapons’s 3rd Biomedical Proficiency Testing.

future may focus on the different mechanisms and behaviors of different nerve agents adduct with cholinesterase, HSA and even DNA in the whole blood. More results about the mechanism of OPNA reaction in human body will promote the development of antidotes.

On the other hand, even so many OPNA biomarkers have been selected in the biological matrix, the sample preparation and the instrument analysis still be complicated and limited in the lab. Fast and accurate detection and analysis for the biological samples of exposure to chemical warfare agents on-site may become a research hotspot with great application prospect.

Summary points

A strong & new evidence that tabun adducts of human serum albumin could be detected by a fluoride ion regeneration process

- The extreme toxicity of organophosphorus nerve agents (OPNA) results from their high affinity and strong ability to inhibit cholinesterase activity by forming a covalent bond with the serine residue. Also, the nerve agents can form a bond with human serum albumin (HSA).
- The OPNA products in the plasma or urine can act as biomarkers for OPNA poisoning and measurement of these biomarkers can help to identify the actual OPNA.
- Previous study suggested that the products resulting from fluoride ion regeneration were from nerve agent adducts to the cholinesterase.
- In this study, we presented a strong evidence that tabun (GA) adducts of albumin could be detected by a fluoride ion regeneration process.

A systematic study of sample preparation & MS behavior for the detection of the products of GA-HSA adducts by fluoride ion regeneration

- The sample preparation contains acetone precipitation, washing, SPE and GC-MS/MS.
- The GC-MS/MS method, precipitation of HSA and washing process, and the SPE operations were optimized to obtain expected results.
- The supernatant after every time of 'acetone precipitation and washing' was collected and detected the residual of GA. The results showed that three-times of 'acetone precipitation and washing' was enough for remove all the GA residue, which avoid the interference.
- The specificity, linearity, relative standard deviation (intraday for $n = 5$ and interday for $n = 10$) and LOD for the method were investigated and achieved expected results.
- Mass spectrometric fragmentation patterns of the some important precursor ions and the transitions for the analytes were presented.

Results & discussion

- We presented a validated method and new evidence for the sensitive and rapid assessment of GA-HSA adducts via fluoride regeneration.
- The methods was successfully applied to identify the biomarkers of nerve agent exposure for the samples in the OPCW's 3rd Biomedical Proficiency Testing. The results were confirmed as completely accurate by OPCW.

Conclusion & future perspective

- This study provides a practical method for the analysis of GA-spiked plasma/HSA samples and a new strategy to verify Chemical Weapons Convention in biomedical samples.
- The results may indicate that different intoxication mechanisms for GA compared with other OPNAs might be due to the difference of -CN and -F from GA and sarin, respectively.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2019-0161

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval. The plasma samples were kindly provided by OPCW with consent of all individual volunteers, and approved by OPCW Ethics Committee.

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