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Improvements in monitoring the N-terminal valine adduct in human globin after exposure to sulfur mustard and synthesis of reference chemicals

Zhiyong Nie, Qin Liu, Jianwei Xie*

Beijing Institute of Pharmacology and Toxicology, No. 27 Tai Ping Road, Hai Dian District, Beijing 100850, PR China

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ABSTRACT

The N-terminal valine adduct (HETE-Val) in globin is believed to behave as a long-lived biomarker after exposure to sulfur mustard (HD). Development of a highly sensitive method for monitoring HETE-Val, particularly at low HD exposure levels or for retrospective detection, would be a significant achievement. In this study, by improving the sample preparation method, a sensitive NCI-GC/MS method was established for the analysis of HETE-Val in globin after HD exposure. To optimize and investigate the sample preparation method, all the relevant HETE-Val chemicals were synthesized, purified, and characterized. By carrying out optimized solid phase extraction (SPE) cleanup followed by modified Edman degradation results in a low detection level and clean baseline. The minimum detectable exposure level of human blood (*in vitro*) to HD is 20 nmol/L (S/N > 3). The interday and intraday precisions of the proposed method were found to be acceptable with less than a 15% relative standard deviation (RSD). A nearly linear dose–effect relationship was observed between HETE-Val and a HD exposure concentration range of 0.1–120 μmol/L. The percentage of HD that reacted with N-terminal valine in globin obtained from human blood (*in vitro*) was quantified using the proposed method.

1. Introduction

Sulfur mustard (HD, 2,2′-dichlorodiethyl sulfide) is a vesicant that can injure skin, eyes, respiratory system, etc. It is one of the most hazardous chemical warfare agents (CWA), and it has been extensively used in World War I and more recent conflicts in the Middle East [1,2]. A number of chemical weapons were abandoned in China by the Japanese army after World War II [3–5]. These weapons contain abundant HD with reactive electrophile molecules and powerful penetrability. HD can attack and react with several targets such as organisms, leaving the victims vulnerable to genetic toxicity, which requires a long recovery period. Skin symptoms after exposure to HD are similar to those of burn and other chemical injuries, and therefore, exact diagnostic methods for HD exposure should be established. Free metabolites in urine and blood can be validated as diagnostic biomarkers of HD exposure, but the major disadvantage lies in their relatively rapid elimination from the body, as ideal biomarkers should be stable and exist for several months after exposure. Covalent adducts with macromolecules, such as protein and DNA, offer the potential for much longer-lived biomarkers of exposure in comparison with free metabolites [6]. These adducts are formed in nucleophilic sites on macromolecules when reacted with HD. They can remain in blood and tissue for a very long period of time and provide definite biomarkers of HD exposure. It has been reported that the N-terminal valine adduct (HETE-Val) in globin is a valuable biomarker that can exist for a long time and still be detected on the 94th day [6,7]. D. Noort et al. used a modified Edman degradation method to determine the HETE-Val content in globin [8,9]. In their method, globin was isolated from human blood and reacted with pentafluorophenyl isothiocyanate (PFPITC). The derivatized N-terminal valine adduct (HETE-Val-P) was then extracted, cleaned using SPE, and analyzed using negative ion GC/MS (NCI-GC/MS) after further derivatization using heptafluorobutyric anhydride or heptafluorobutyryl imidazole (HFBI). The method has a minimum detection limit of 100 nmol/L. Some modifications have been developed in an attempt to lower the minimum detectable concentration, but the results have been unsatisfactory [8,9].

As is well known, highly sensitive bioanalytical methods not only allow the detection of low levels of exposure but also lengthen the time window for retrospective detection [10]. In this report, we focused on improving the sensitivity of the detection method of HETE-Val in globin. For this purpose, all the relevant chemicals were synthesized, such as HETE-Val and HETE-Val-P, to optimize the SPE clean-up procedure. The HFBI derivatization of HETE-Val-P was also carried out to investigate the derivatization efficiency. The improved methodology lowered the detection levels from the

* Corresponding author. Tel.: +86 10 68225893; fax: +86 10 68225893.
E-mail addresses: niezhiyong2008@163.com (Z. Nie), liuq920@yahoo.com.cn (Q. Liu), xiejw@nic.bmi.ac.cn, ammslta@gmail.com (J. Xie).

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previously reported limit of detection (LOD) of 100 nmol/L (HD to blood, *in vitro*) [8,9] to 20 nmol/L. The SPE recoveries were also satisfactory.

2. Experiments

2.1. Materials

HD (96% purity) was provided by the Institute of Chemical Defense of CPLA. L-Valine and pentafluorophenyl isothiocyanate (PFPITC) were purchased from Sigma Chemical Co. (St. Louis, USA). The Florisil used in this experiment came from Alletch (mesh: 60–100, PA, USA). Heptafluorobutyryl imidazole (HFBI) was obtained from TIC (Tokyo, Japan). The toluene, dichloromethane, ethyl acetate and acetonitrile used were of HPLC grade and obtained from J.T. Baker (PH, USA). 1,2-Dichlorodeuterethane was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The other chemical reagents used were of analytical grade and obtained from Sinopharm Chemical Reagent Co. (Beijing, China). Human blood was provided by Hospital 307 (Beijing, China).

2.2. Instrumentation

GC–MS analysis was performed on an Agilent 5975 mass selective detector interfaced to an Agilent 6890N GC with an Agilent 7683B autoinjector (Agilent, USA). The GC column was a 25-m HP-5MS capillary column (i.d. of 0.2 mm and film thickness of 0.33 μm). Helium was used as the carrier gas in the constant flow mode (0.8 mL/min). The oven temperature was initially held at 120 °C for 2 min, was increased to 280 °C at a rate of 20 °C/min, and was finally maintained at 280 °C for 5 min. Splitless injections of 1-μL in volume were made using an autosampler. The splitless time was 0.5 min. The injector and transfer-line temperatures were 260 °C and 280 °C, respectively. An MS analysis was conducted using negative chemical ionization (NCI) with methane as the reagent gas. The source and quadrupole temperatures were both set at 150 °C. Ions at an m/z of 564 ($M^{-\bullet}$ – 3 HF, analyte) and 568 ($M^{-\bullet}$ – 3 HF, internal standard) were monitored using the selected ion monitoring (SIM) mode. The other equipment used included a CentriVap concentrator (Labconco, America), rotary evaporator (Yarong, China), WRS-2A melting point apparatus (Jingke, China), JNM-ECA 400 MHz NMR spectrometer (JEOL, Japan), 5890 GC-FID (Agilent, USA), and Micromass LC–TOF MS (Micromass, England).

2.3. Synthesis of reference chemicals and 2-(2-chlordeuterethylthio) chloroethyl (HD-d_4)

The synthetic routes of the reference chemicals used are shown in Scheme 1. Preparations of the half-mustard and HETE-Val were similar to those published by M. Kameswara Rao [11] except for a few modifications to improve the purity of the products.

2.3.1. 2-(2-Chloroethylthio) ethanol [half-mustard] [11]

A solution of sodium methoxide, prepared by adding 2.3 g (0.1 mol) of small slices of sodium to 60 mL of dry methanol, was added dropwise to 2-mercaprtoethanol (7 mL, 0.1 mol) and stirred. The stirring was continued for about 30 min, and 1,2-dichloroethane (60 mL) was then added. The mixture was maintained at 4 °C overnight. The precipitated sodium chloride was then filtered off before the solvents were removed using a rotary evaporator. 120 mL of ether was added to the residue followed by washing with water. The half-mustard thus was obtained and stored along with anhydrous magnesium sulfate at −20 °C until required. The identification and purity check (>98%) of the half-mustard was carried out using GC/MS.

2.3.2. N-(2-hydroxyethylthioethyl)-L-valine [HETE-Val] [11,12]

L-Valine (0.5 g, 0.0043 mol) was dissolved in 4 mL of a 0.5 mol/L sodium hydroxide solution heated to about 60 °C. A half-mustard ether solution (0.1 g/mL) was gradually added while stirring. The pH of the reaction mixture was adjusted to about 10 (monitored by adding thymolphthalein) by adding a 2 mol/L sodium hydroxide solution. When the reaction was completed (the reacting solution stopped the reddening of ninhydrin), about 1 g of half-mustard (0.007 mol) was added, and the aqueous solution was washed with 3 portions of dichloromethane (5 mL). The solution was then adjusted to pH 2.7 by the addition of concentrated hydrochloric acid (about 0.4 mL) and was shaken with 2 portions of ether (5 mL). The water fraction was removed using a rotary evaporator and the solid residual was washed with isopropyl alcohol (10 mL ×2). The solid was dissolved in 80 mL of boiling isopropyl alcohol. White crystallized precipitate was obtained during the cooling stage and recrystallized with boiling isopropyl alcohol. Finally, 0.2 g of product was yielded with m.p. 216–217 °C. The purity (>97%) was checked using LC–TOF MS. The product was also characterized based on ^{1}H, ^{13}C NMR and mass spectral data.

2.3.3. Reaction product of HETE-Val and Edman reagent

HETE-Val (20 mg) was dissolved in a potassium bicarbonate solution (4 mL, 0.25 mol/L). Acetonitrile (2 mL) containing PFPITC (20 μL) was added and mixed using a vortex agitator. The mixture was heated for 2 h at 45 °C. The product was extracted with ether (3 mL × 3), and then washed with water (3 mL × 2). The ether layer was dried with anhydrous $MgSO_4$ and then evaporated to dryness under a nitrogen stream, while the residue was evaporated in a centrifugal concentrator for 90 min at 60 °C to remove any low boiling point impurities. The reaction product, HETE-Val-P, was obtained (27 mg) and characterized based on ^{1}H, ^{13}C NMR and mass spectral data. The purity (>97%) was checked using a GC-FID.

2.3.4. HFBI derivatization of HETE-Val-P

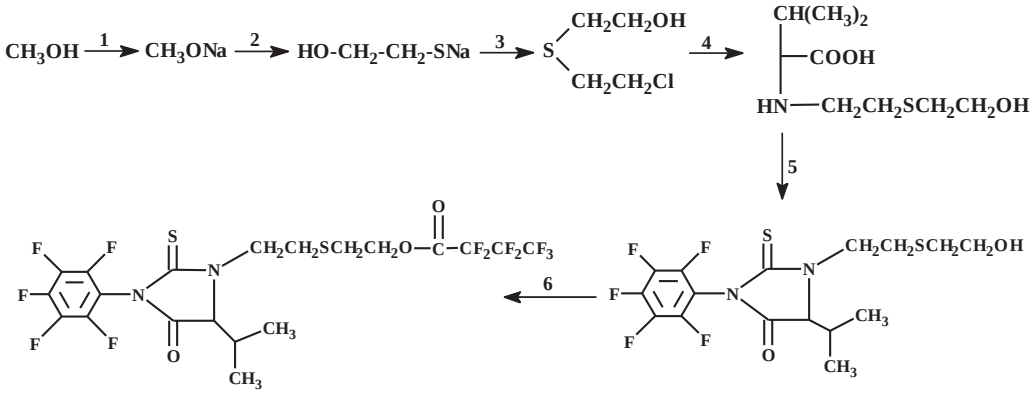
The HETE-Val-P product was dissolved in 1 mL of toluene. Then, 1 mL of toluene containing HFBI (200 mg) was added and mixed. The mixture was heated at 60 °C for 0.5 h. The HFBI derivative was washed with water (2 mL × 2) and dried with anhydrous $MgSO_4$. Then, after removing the solvent, the product (HETE-Val-P-P) was obtained and analyzed. The purity (>96%) was checked using a GC-FID.

2.3.5. 2-(2-Chlordeuterethylthio) chloroethyl (HD-d_4)

3 mL of 2-hydroxyethylthiosodium solution (prepared as described above) was added dropwise to 1,2-dichlorodeuterethane (2 g) under 0 °C. The mixture was kept at 4 °C overnight. The precipitated sodium chloride was then filtered off, and the solution was evaporated to remove the solvent under 60 °C. 15 mL of ether was added and washed with water. 2-(2-Chlordeuterethylthio) ethanol was thus obtained and dried with anhydrous magnesium sulfate. The compound was identified using GC/MS and then chloridized using sulfurous oxychloride at 80 °C. When the reaction was completed and cooled to room temperature, the solvent was removed in a centrifugal concentrator, and 150 mg of HD-d_4 was finally achieved. An identification and purity check of the product was carried out using GC/MS, which revealed a purity of more than 96%, with no detected HD-d_0.

2.4. Preparation of internal standard solution for the analysis of HETE-Val

50 μL of 0.01 mol/L HD-d_4 in a CH_3CN solution was added to human blood (5 mL) and mixed. After incubation at 37 °C for 2 h, the globin was isolated according to the procedure described by Bailey et al. [13]. Using centrifugation (1500 × g, 10 min), the plasma was



1 Na, 2 HO-CH_2-CH_2-SH, 3 Cl-CH_2-CH_2-Cl, 4 L-valine, 5 PFPITC, 6 HFBI

Scheme 1. Schematic of synthetic route of related reference chemicals.

separated from the erythrocytes, which were washed twice with physiological saline and hemolyzed in water (5 mL). Cell debris was removed through centrifugation (20,000 × *g*) and the globin was precipitated from the supernatant when 1% HCl in acetone was added. The protein was washed with 1% HCl in acetone, acetone, and lastly ether. Finally, the globin was dried and dissolved in formamide (20 mg/mL), which was used as the internal standard [8,9] for the analysis of HETE-Val throughout the experiments. Globin alkylated with a concentration series of HD in CH_3CN solutions was prepared in a similar manner.

2.5. Preparation of calibrated standard solutions

A series of 0, 1.5, 5.0, 25.0, 100.0, 500.0, 1200.0 μg/L (0–5.430 μmol/L) HETE-Val formamide solutions were prepared. Then, the HETE-Val solutions (50 μL), internal standard solution (50 μL, prepared as discussed in Section 2.4), pyridine (8 μL), and PFPITC (8 μL) were added to 2 mL of formamide. The reaction temperature was maintained at 60 °C for 2 h. After cooling to room temperature, the mixture was then extracted using toluene (1 mL) and centrifuged for 5 min (3000 rpm). The extraction was repeated twice, and the organic layers were combined and washed with water (0.5 mL × 2), Na_2CO_3 solution (0.1 mol/L, 0.5 mL), and water (0.5 mL). The toluene layer was dried with anhydrous $MgSO_4$, then evaporated to dryness under nitrogen stream, and dissolved in 100 μL of toluene. After that, a Florisil cartridge (500 mg/3 mL) was packed and conditioned with ethyl acetate/dichloromethane (1:9, v/v; 4 mL) followed by dichloromethane (2 mL). The toluene solution was applied on the cartridge and washed with dichloromethane (2 mL) and ethyl acetate/dichloromethane (1:9, v/v; 1 mL). The analyte was then eluted with ethyl acetate/dichloromethane (1:9, v/v, 2 mL). The latter eluate was evaporated to dryness and dissolved in toluene (200 μL). Toluene (70 μL) containing HFBI (14 mg) was added, and the derivatization was completed at 60 °C for 30 min. After cooling, the reaction mixture was washed twice with water (200 μL), Na_2CO_3 aqueous solution (0.1 mol/L, 200 μL), and again with water (200 μL). The toluene layer was evaporated to dryness under a nitrogen stream after drying ($MgSO_4$), dissolved in toluene (50 μL), and then analyzed using GC/MS.

A series of 2.5, 10.0, 50.0, 300.0, 800.0 μg/L (0.011–3.620 μmol/L) HETE-Val formamide solutions were prepared as described above. The intraday precision of the method was assessed through three replicated analyses for five HETE-Val concentration levels, while the interday precision was assessed through a five-day study for three HETE-Val concentration levels.

2.6. Preparation procedure for samples used in GC/MS analysis

The preparation of the samples was similar to that reported previously [8,9] except for the SPE process. Globin (20 mg) isolated from human blood exposed to HD was dissolved in formamide (2 mL). An internal standard solution (50 μL), along with pyridine (8 μL) and PFPITC (8 μL), was then added, following the preparation method described in Section 2.5.

2.7. Recovery of SPE process and derivatization efficiency

To assess the recovery of HETE-Val-P from the SPE cartridge, a matrix was first prepared as described in Section 2.6 before the SPE procedure was conducted, except that human blood was not exposed to HD. The synthesized HETE-Val-P product was then added into the matrix, which was followed by the SPE steps. The equimolar HETE-Val-P was also added into the SPE cleaned matrix. After analyzing using a GC/MS, the recovery was calculated by comparing the peak areas from each addition.

The derivatization efficiency was determined as follows. The HETE-Val-P was derivatized and concentrated as described in Section 2.5. The equimolar HETE-Val-P-P solution was also dealt with using a similar process except for the derivatization stage, and the derivatization efficiency was then obtained by comparing the peak areas.

All of the experiments were carried out safely. The operators all wore protective devices. The ventilating system was in good condition, and all residues from the experiments were immediately and completely decontaminated.

3. Results

3.1. Characterization of synthesized reference chemicals and HD-d_4

The reactant ratio needs to be adjusted to obtain a purer product during the synthesis of HETE-Val. If an excessive amount of half-mustard is added, too many by-products will be obtained. The main by-product is the reaction product of two half-mustard molecules reacting with one valine molecule, which is difficult to separate from the target compound. To reduce the amount of by-products, the reacting process in our experiment was monitored using ninhydrin. When the reacting solution no longer reddened the ninhydrin, the reaction was deemed complete. The product was recrystallized twice to improve the purity (>97%). The HETE-Val was characterized using ^1H and ^{13}C NMR and HRMS data (Table 1), which were well-matched with the molecular structure [11,12].

Table 1
Mass spectrum and NMR data for synthesized chemicals.

Chemicals	MS and NMR spectral data
HD-d_4	MS (EI): m/z 162 (M^+), 127 ($M-Cl)^+$, 113 ($M-CH_2Cl)^+$
HETE-Val	MS (ESI-TOF/MS): m/z 222.1 $(M+H)^+$, 244.0 $(M+Na)^+$, 260.0 $(M+K)^+$ ^{1}H NMR (D_2O) δ (ppm): 0.88 (3H, d, CH_3), 0.92 (3H, d, CH_3), 2.10 (1H, m, $CH(CH_3)_2$), 2.61 (t, 2H, SCH_2CH_2OH), 2.78 (2H, m, $NHCH_2CH_2S$), 3.15 (2H, m, $NHCH_2$), 3.39 (d, 1H, NHCH), 3.62 (2H, t, CH_2OH) ^{13}C NMR (D_2O) δ (ppm): 18.98, 19.72 (2 × CH_3), 28.55 (SCH_2CH_2NH), 30.82 ($CH(CH_3)_2$), 34.77 ($HOCH_2CH_2S$), 48.12 ($HNCH_2$), 61.73 (CH_2OH), 69.72 (CHNH), 173.86 (COOH)
HETE-Val-P	MS (EI): m/z 428 ($M^{+\bullet}$), 410 $(M-H_2O)^+$, 383 $(M-CH_2CH_2OH)^+$, 351 $(M-SCH_2CH_2OH)^+$ ^{19}F NMR ($CDCl_3$) δ (ppm): aromatic fluorines 141.7 [1F, d, ortho], 142.1 [1F, d, ortho], 149.9 [1F, t, para], 160.7 [2F, m, meta] ^{1}H NMR ($CDCl_3$) δ (ppm): 0.99 (3H, d, CH_3), 1.27 (3H, d, CH_3), 2.44 (1H, m, $CH(CH_3)_2$), 2.84 (t, 2H, SCH_2CH_2OH), 2.85 (1H, m, $NHCH_2CH_2S$), 3.07 (1H, m, $NHCH_2CH_2S$), 3.66 (1H, m, $NHCH_2$), 3.82 (2H, t, CH_2OH), 4.35 (d, 1H, NHCH), 4.38 (1H, m, $NHCH_2$) ^{13}C NMR (D_2O) δ (ppm): 15.49, 17.29 (2 × CH_3), 28.40 (SCH_2CH_2N), 29.42 ($CH(CH_3)_2$), 35.17 ($HOCH_2CH_2S$), 44.92 (NCH_2), 60.84 (CH_2OH), 67.93 (CHN), 136.62–145.60 (aromatic carbons), 169.97 (CON), 179.74 (CSN)
HETE-Val-P-P	MS (EI): m/z 624 ($M^{+\bullet}$), 605 $(M-F)^+$, 383 $(M-CH_2CH_2O_2C_4F_7)^+$, 351 $(M-SCH_2CH_2O_2C_4F_7)^+$, 241 $(CH_2CH_2O_2C_4F_7)^+$ MS (NCI, methane): m/z 624 (M−), 604 (M−HF), 584 (M−2HF), 564 (M−3HF), 544 (M−4HF), 524 (M−5HF), 504 (M−6HF) ^{1}H NMR ($CDCl_3$) δ (ppm): 0.99 (3H, d, CH_3), 1.27 (3H, d, CH_3), 2.42 (1H, m, $CH(CH_3)_2$), 2.94 (1H, m, $NHCH_2CH_2S$), 2.96 (m, 2H, SCH_2CH_2OH), 2.99 (1H, m, $NHCH_2CH_2S$), 3.68 (1H, m, $NHCH_2$), 4.28 (d, 1H, NHCH), 4.36 (1H, m, $NHCH_2$), 4.58 (2H, t, CH_2OCO) ^{13}C NMR (D_2O) δ (ppm): 15.54, 17.30 (2 × CH_3), 28.79 (SCH_2CH_2N), 29.56 ($CH(CH_3)_2$), 29.88 ($HOCH_2CH_2S$), 44.79 (NCH_2), 66.76 (CH_2OH), 67.92 (CHN), 104.63–118.76 (3C, heptafluoroisopropyl carbons), 136.73–145.72 (6C, aromatic carbons), 158.18 (CON), 169.89 (COO), 179.98 (CSN)

During the synthesis of the HETE-Val-P, we noticed that 1-propanol in the reaction solution, which had been used in previously published methods [11,12], reacted with PFPITC and yielded by-products that were difficult to remove. When acetonitrile was used instead of 1-propanol, the target HETE-Val-P product could be depurated easily. The HETE-Val-P was characterized using ^{1}H, ^{13}C and ^{19}F NMR and mass spectral data (Table 1). The ^{1}H NMR data led us to the conclusion that the H atoms covalently linked to the same C atom of the HETE-Val-P were severely split. The purity (>97%) of the HETE-Val-P was satisfactory as determined through a direct analysis using a GC-FID.

The microsynthesis of 2-(2-chlordeuterethylthio) ethanol (half-mustard-d_4) was similar to the synthesis of the half-mustard except that the reaction temperature should be restricted below 0 °C to reduce the amount of by-products. The half-mustard-d_4 obtained was reused to achieve HD-d_4. The purity of the final HD-d_4 product was larger than 96% as determined through GC/MS. The major impurity (3%) was the half-mustard-d_4, and the deuterium labeling ratio was nearly 100%.

3.2. Improvement of SPE procedure

A large amount (up to 500 mg) of globin had been used to increase the sensibility of the analyte [8], but the impurities coexisting in the sample prevented a proper analysis, making it necessary to optimize the SPE conditions to obtain purer analytes. For this purpose, we spiked HETE-Val-P into the matrix, and then applied the mixture onto an SPE cartridge to investigate the effectiveness of the SPE procedure. During the process of selecting the SPE cartridge, the cleanup efficiency and analyte recovery should be taken into consideration. Two sorbent types of silica gel and Florisil were investigated. Although the silica gel cartridge could effectively eliminate matrix interference, the recovery was not satisfactory, particularly at lower concentrations, as some of the products were adsorbed completely and elution did not occur. The Florisil cartridge was selected for further optimization. Several types of eluting solvents were tested. The results showed that ethyl acetate, ether, toluene, anisole, dichloromethane, and acetonitrile could separate HETE-Val-P from the matrix, but methanol had difficulty in achieving separation. The separation abilities of the mixture solvents were then compared and their mixed ratios were also investigated. Finally, the optimized SPE condition was established as described in Section 2.5, under which cleaner baselines and good analyte recovery could be achieved. The SPE recoveries (54–56%), expressed as a percentage, were investigated as the ratio of peak areas in HETE-Val-P-P after SPE and direct HETE-Val-P derivatization. The results are shown in Table 2 (n = 3).

Table 2
SPE recoveries of the method.

Parameters	HETE-Val-P (nmol/L)		
	30	300	1500
Recoveries (%)	56.3	54.6	54.1
RSD (%)	11.5	10.4	14.0

3.3. Linearity and precision of the method

A series of calibrated standards for quantifying the amount of HETE-Val in globin were prepared at various concentrations using a fixed internal standard concentration. These solutions were analyzed using NCI-GC/MS. The calibration curves obtained were linear over a concentration of 0–1200.0 μg/L, with a correlation coefficient, R^2, of over 0.999. The precision was measured by calculating the relative standard deviation (RSD) of each group of the HETE-Val concentration levels. The results are shown in Table 3. For all interday and intraday studies, the RSD was less than 15%.

3.4. Detection of HD exposure in human blood and limits of detection

By optimizing the SPE conditions, the lower minimum detectable exposure (20 nmol/L, S/N > 3) level of human blood (*in vitro*) to HD was obtained. The limit of quantitation (LOQ) was 100 nmol/L (S/N > 10, Fig. 1). The results of a representative analytical run (1 μmol/L HD exposure) are also shown in Fig. 1. The ultimate volume of analyte used in our method is 50 μL, compared

Table 3
Interday and intraday precisions of the method.

HETE-Val (μg/L)[a]	Interday (n = 3)		Intraday (n = 5)	
	C_1[b]	RSD (%)	C_2[b]	RSD (%)
2.5	2.7	5.3		
10.0	11.2	5.0	11.5	5.7
50.0	48.1	1.6	48.4	2.5
300.0	340.1	1.4	339.0	5.6
800.0	809.0	2.1		

[a] The five concentrations equal to 0.011, 0.045, 0.226, 1.358, and 3.620 μmol/L, respectively.
[b] Detected concentration (μg/L).

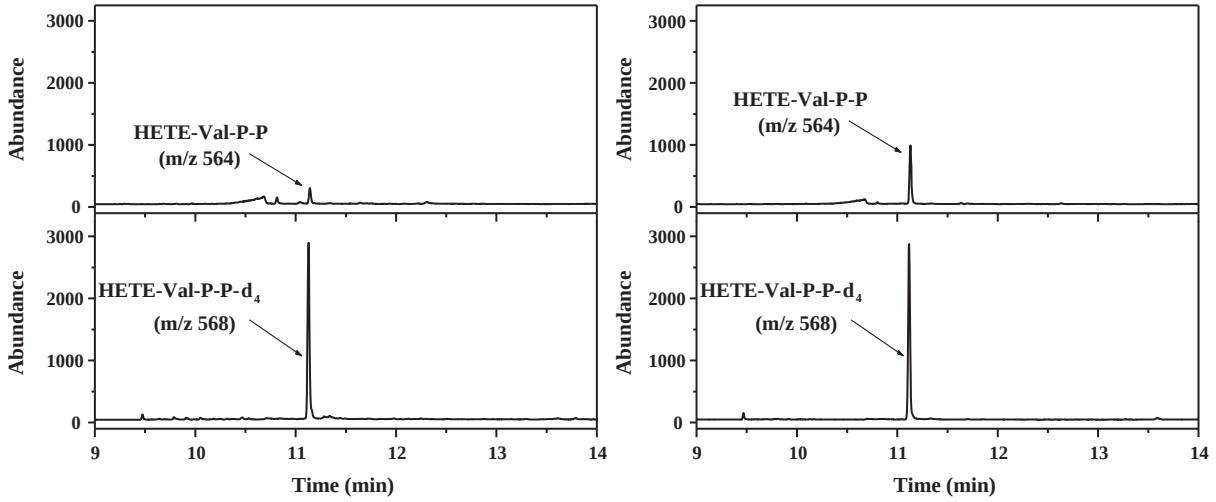


Fig. 1. Representative NCI-GC/MS analysis of HETE-Val in globin (20 mg) isolated from human blood exposed to 100 nmol/L (left) and 1 μmol/L (right) of HD. The upper panels represent the analyte (*m*/*z* 564), and the lower panels represent the internal standard (*m*/*z* 568) in each chromatogram.

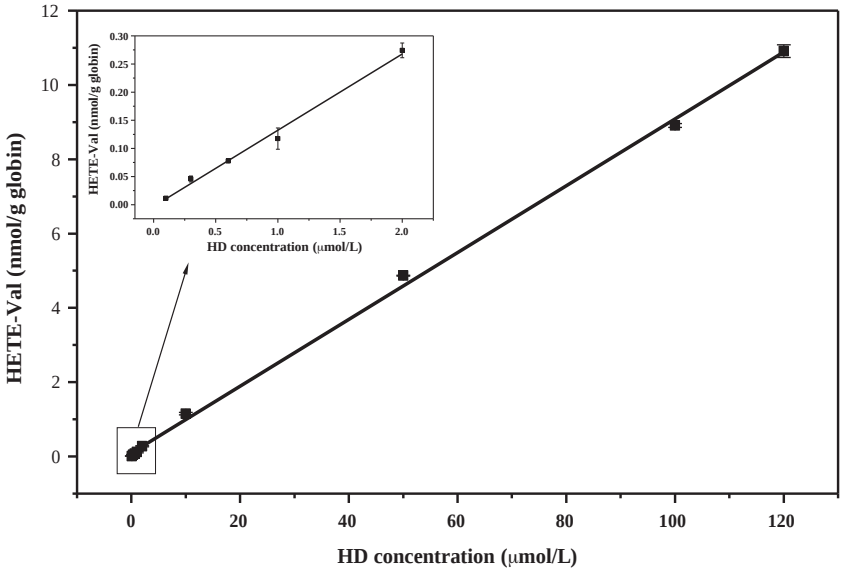


Fig. 2. Relationship between HD concentrations and analytes (mean ± sd, *n* = 3).

with 30 μL used in other reported methods [8,9], which suggests that the sensitivity was improved by almost tenfold through our method. The baseline of the chromatogram was cleaner. Even when the volume of the final dilution solvent was reduced, or the amount of globin was increased, despite a noise intensity and unclean baseline, the analyte signal was still reinforced and the S/N ratio still increased. In other words, the minimum detectable exposure level can be reduced even further.

Globin samples isolated from human blood, which had been exposed to various concentrations of HD (0.1–120 μmol/L), were analyzed according to the procedure based on NCI-GC/MS analysis after a modified Edman degradation. It should be noted that the data obtained directly from the calibration curve are expressed in μg/L. Through conversion, the HETE-Val liberated from the globin after Edman degradation were obtained as moles/gram. The results are presented in Fig. 2, which shows a nearly linear dose–effect ($R^2 > 0.99$) relationship between HEVE-Val and HD exposure concentrations. Since about 0.14 g of globin could be isolated from 1 mL of blood in our experiments, which is close to the average globin content in human blood [14], the amount of moles of HEVE-Val per mL of blood were obtained. Therefore, the percentage of HD that reacted with the N-terminal valine in globin was investigated within this HD exposure concentration range, as shown in Table 4.

3.5. HFBI derivatization efficiency and response of HETE-Val-P-P based on GC/MS

The HFBI derivatization efficiencies (90–92%) were satisfactory as shown in Table 5 ($n = 3$). The LOD (1 nmol/L, S/N > 3) and LOQ (3 nmol/L, S/N > 10) were also obtained through the direct injec-

Table 4
Percentage of HD that reacted with N-terminal valine in globin.

HD (μmol/L)	HETE-Val[a]	RSD (%)	HETE-Val[b]	Percent[c]
0.6	0.078	1.7	0.011	1.8
2.0	0.27	4.8	0.038	1.9
10.0	1.15	2.8	0.161	1.6
50.0	4.87	0.2	0.682	1.4

[a] HETE-Val content in globin (nmol/g globin).
[b] HETE-Val content in blood (nmol/mL blood, based on 0.14 g of globin per mL of blood).
[c] Percentage.

Table 5
HFBI derivatization efficiencies.

Parameters	HETE-Val-P (nmol/L)		
	15	300	1500
Efficiency[a] (%)	91.6	90.0	92.1
RSD (%)	7.7	5.9	6.6

[a] HFBI derivatization efficiency.

tion of a 1 μL synthesized compound of HETE-Val-P-P in a GC/MS system.

4. Conclusions

A sensitive NCI-GC/MS method was established for the analysis of HETE-Val in globin after HD exposure by improving the sample preparation method. For this purpose, all the relevant HETE-Val chemicals were synthesized, purified, and characterized. The SPE cleanup procedure was optimized, and the recoveries (54–56%) were investigated. The optimized SPE procedure resulted in low detection levels and clean baselines, and thus, a low minimum detectable exposure (20 nmol/L, S/N > 3) level of human blood (*in vitro*) to HD was obtained. A satisfactory HFBI derivatization efficiency (90–92%) was also obtained in this procedure.

The HETE-Val contents in globin of human blood were quantified after HD exposure (*in vitro*), and the relationship between HD exposure levels and analytes showed a nearly linear dose–effect curve. The percentage of HD that reacted with the N-terminal valine in globin was about 1–2%. Thus, it is possible to estimate the HD exposure levels in humans or animals according to the amount of HETE-Val in globin by using the established methodology.

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