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Determination of ethylene oxide–hemoglobin adduct by silylation and gas chromatography–electron impact-mass spectrometry

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Abstract

A gas chromatography–electron impact ionization-mass spectrometric (GC–EI-MS) assay was developed for the determination of ethylene oxide–hemoglobin adduct (*N*-2-hydroxyethylvaline, HEVal). HEVal and deuterated HEVal (d_4-HEVal) were synthesized for identification and quality control. Globin samples were separated from red blood cells (RBCs) by acidic isopropanol and extracted with ethyl acetate. HEVal adduct in globin was transformed to HEVal-pentafluorophenylthiohydantoin derivative by modified Edman-degradation method, which was extracted from globin with diethylether. d_4-HEVal was used as an internal reference standard. The dried extract was derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBDMSTFA)-NH_4I (1000:4, w/w) containing 0.4 mg of dithioerthritol. The TBDMS derivative of HEVal had very good chromatographic property and offered sensitive response of GC–EI-MS. The recovery of HEVal was about 81.6% and the coefficient of variation was 5.0% at the concentration of 311 pmol/g. Low limit of detection (LOD) of the assay was 1.8 pmol/g in 0.1 g hemoglobin. The experiments have demonstrated to detect background level of HEVal adduct in human blood. HEVal adduct in globin was detected between 12 and 6573 pmol/g.

Keywords: EO–hemoglobin adduct; Hydroxyethylvaline; GC–EI-MS; Silylation

1. Introduction

Ethylene oxide (EO, CAS No. 75-21-8) is colorless vapor at ambient temperature and industrially important chemical with various applications, for example, the production of glycol, glycol ethers, non-ionic surfactants and polyacrylamides. EO is exposed to human by inhalation of contaminated air at work places where EO is used. The additional sources of occupational exposure are sterilization plants and sterilization facilities in hospitals.

Long-term inhalation studies performed in mice and rats have resulted in development of malignant tumors at multiple sites [1–3]. In human, occupational exposure to EO has increased levels of chromosomal aberrations, sister chromatid exchanges and micronuclei in blood cells [1]. EO has been classified as a human carcinogen (Group 1) by the IARC [1]. The Dutch occupational exposure limit (OEL) for EO is 0.92 mg/m^3 (0.5 ppm) as the 8-h time-weighted average (8-h TWA)[4], and U.S. and German OEL are 1.8 mg/m^3 (1 ppm) as the 8-TWA and 9.2 mg/m^3 (5 ppm) as the 15 min-excursion [5,6]. Otherwise, UK permissible exposure limit for EO is 9.2 mg/m^3 (5 ppm) [7].

EO is a direct alkylating agent, which attacks at the various nucleophilic sites of macromolecules, such as globins and DNA bases [8]. The measurement of hemoglobin and DNA adducts is a useful tool for monitoring exposure to electrophilic agents. EO-induced protein adduct, the N-terminal hydroxyethyl valine (HEVal) of hemoglobin has been validated as a biologically effective dose marker for several animal species exposed to EO [9–13]. At the current OEL for EO the biological exposure limit (BEL) is 3.2 nmol HEVal/g globin [14].

It is important to have techniques available, which allow for the accurate analysis at low pmol/g levels of HEVal in hemoglobin. Until now, gas chromatographic methods

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have been published for the analysis of HEVal adduct in hemoglobin, involving electron impact ionization-mass spectrometry (GC–EI-MS) [14–17], negative-ion electron-capture ionization-mass spectrometry (GC–NICI-MS) [12,13,17–23] and using negative-ion chemical ionization-tandem mass spectrometry (GC–NICI-MS–MS) [24].

Although NICI-MS methods have enough sensitivity for pentafluorophenylthiohydantoin (PFPTH) derivative produced by using a modified Edman-degradation method, a new sensitive method needs to develop monitoring method using relatively cheap and common instrument such as GC–EI-MS. GC–EI-MS has insufficient sensitivity to monitor background concentrations of HEVal adduct in human blood samples. Until now, silylation method has been used to improve the sensitivity and chromatographic property of GC–EI-MS.

This paper describes the silylation method of PFPTH-HEVal by using various silylating reagents, and subsequently the detection by GC–EI-MS.

2. Experimental

2.1. Chemicals and reagents

Ethylene oxide, bromoisovaleric acid, trimethylsilyl imidazole (TMS-I), ammonium iodide (NH_4I), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBDMSTFA), formamide, *d*10-pyrene (M_{wt} = 212) and Dowex-50 ion exchanger were purchased from Sigma & Aldrich (St. Louis, MO, USA). 2-Aminoethanol was purchased from Across-Organic (Geel, Belgium), pentafluorophenylisothiocyanate (PFPITC) from Fluka (Buchs, Swizerland), d_4-2-aminoethanol from CDN (Quebec, Canada) and sodium heparin from Choong-Wae Phama Corp. (Korea, Seoul).

Analytical grade of sodium carbonate, sodium hydroxide, sodium sulfate, aqueous ammonia, hydrochloric acid were purchased from Aldrich (St. Louis, MO, USA). Diethyl ether, toluene, acetone, ethyl acetate and isopropanol (Merck, Darmstadt, Germany) were used as solvents.

2.2. Syntheses of standards

Synthesis of HEVal was performed according to the method described by Rydberg et al. [25]. 2-Bromoisovaleric acid (540 mg, 2.98 mmol), 2-aminoethanol (1430 μl, 23.75 mmol) and water (0.36 ml) were added into a glass-stoppered test tube. The mixture was reacted in incubation overnight at 37 °C and cooled to room temperature. Following precipitation with 18 ml of acetone and centrifugation, the residue was dissolved in 3.6 ml of 1 M HCl and purified on a Dowex-50 ion exchanger, which was subsequently washed with water and eluted with 22 ml of 2 M aqueous ammonia. After evaporation to dryness, the residue was diluted with distilled water and crystallized from acetone–ethanol (2:1, v/v).

Synthesis of d_4-*N*-2-hydroxyethyl valine (d_4-HEVal, internal standard) was synthesized by above explained procedure except the using of d_4-2-aminoethanol instead of 2-aminoethanol.

2.3. Collection of blood sample and isolation of globin

Blood samples (1–1.2 ml) were obtained by using disposable syringe made of polypropylene heparinized with sodium heparin. After collection red blood cells (RBCs) were separated from blood by centrifugation at 1500 × *g*, for 20 min at 4 °C. The RBCs were washed with cold 0.9% sodium chloride and centrifuged. The packed RBCs were stored at −80 °C until analysis.

Globin was isolated by the method of Mowrer et al. [26] and Carmella et al. [27]. The washed RBCs were thawed at room temperature, suspended in distilled water (1 ml water/1 ml RBC), vortexed gently and incubated for several minutes (10–20 min) at room temperature. 0.5% HCl in cold isopropanol was added to dissociate the heme group from globin. The mixture was centrifuged at 1500 × *g*, for 30 min at 4 °C. The supernatant was transferred to a clean Nalgene centrifuge tube and globin was precipitated by addition of ethyl acetate followed by centrifugation at 1500 × *g*. The globin was washed twice with fresh ethyl acetate and once with *n*-pentane, dried under stream of nitrogen at room temperature and then transferred to a dessicator under vacuum overnight. The dried globin samples were stored at −80 °C until analysis.

2.4. Edman-degradation method

HEVal was separated using a modified Edman-degradation method of Törnqvist et al. [28]. About 0.1 g of globin was added in glass tube and dissolved with 2 ml of formamide. The globin was mixed to complete dissolution and then neutralized by addition of 1N NaOH. Internal standard, d_4-HEVal and 14 μl of degradation reagent (PFPITC) were added to the solution. The solutions were left over night in a shaking mixer in the dark at room temperature and finally warmed at 45 °C for 90 min. Two milliliters of purified water was added to the solutions, which were subsequently extracted twice with 4 ml of diethyl ether. The combined ethereal phases were evaporated to dryness by a gentle stream of N_2 and the residue was redissolved in toluene. The toluene solution was subsequently extracted with 2 ml aliquots of purified water, 0.1 M aqueous sodium carbonate solution (twice) and with water again. The toluene extract was evaporated to dryness at 50 °C in a heating block using a gentle stream of N_2 and finally in a desiccator over P_2O_5–KOH for at least 30 min, before derivatization.

2.5. Derivatization

A dry residue of the extract from Edman-degradation method was dissolved with 50 μl of silylating reagents. The tubes were heated for 60 min at 60 °C in 100% MSTFA and for 120 min at 80 °C in 100% MTBDMSTFA, and for 90 min at 80 °C in MTBDMSTFA + 0.4% NH_4-I + 0.8% dithioerythritol (w/w/w). After silylation, a 1 μl of the solution was injected directly in the GC system.

The reaction rates of HEVal with the derivatizing reagents were studied as following: 50 μg of HEVal were, respectively, added in 10 test tubes, and the solutions were dried. Ten dried residues in test tubes were dissolved with the silylating reagent,

and heated to the studied temperature. This solution was analyzed at reaction times of 5, 15, 30, 45, 60, 75, 90, 105 and 120 min. After the cooling of the solution, 200 μg of *d*10-pyrene dissolved in acetonitrile was added in the solution, and a 1 μl of the solution was injected directly in the GC system. The ratio of peak area of silylated product to that of *d*10-pyrene was used for the calculation of the reaction rate of HEVal with the derivatizing reagents. *d*10-Pyrene was used as internal standard for the measurement of the reaction rate, because it was not derivatized with the silylating reagent.

2.6. Instruments

Apparatus ^{1}H NMR spectra were recorded on a 300 spectrometer (Me4Si as internal standard).

All mass spectra were obtained with a 6890GC/5973N MSD (Agilent Technologies, Palo Alto, CA, USA). The ion source was operated in the electron ionization mode (EI 70 eV, 230 °C). Full-scan mass spectra (*m*/*z* 40–800) were recorded for analyte identification. Separation was achieved with an HP fused-silica capillary column with cross-linked 5%-phenyl methylsilicone (HP-5MS), 30 m length, 0.25 mm i.d., 0.25 μm film thickness. Samples were injected in the pulse splitless mode. The flow-rate of the helium was 1.0 ml/min. The operating parameters were as follows: injector temperature, 280 °C; transfer line temperature, 280 °C; oven temperature, programmed from 100 °C (held for 1 min) at 10 °C/min to 280 °C (held for 5 min). The ions selected for quantification by SIM were *m*/*z* 467 and *m*/*z* 425 for TBDMS-PFPTH-HEVal and *m*/*z* 471 and *m*/*z* 429 for TBDMS-PFPTH-d_4-HEVal (ISTD).

2.7. Calibration and quantification

Calibration curve for the analyte was established by extraction and derivatization after adding 0.6, 1.2, 3.1, 15.6, 31.1, 62.1, 124.2, 1242 and 2484 pmol of HEVal and 1555 pmol of d_4-HEVal in 0.1 g of control globin, in which the concentration of HEVal was identified below MDL. The ratio of peak area of analyte to that of ISTD was used in the quantification of EO–Hb adduct.

3. Results and discussion

3.1. Syntheses of HEVal and d_4-HEVal

The final product was crystallized from acetone–ethanol (2:1, v/v) to yield 0.75 mmol (about 25%) as white crystals. NMR showed peaks at δ 0.887, 0.927 [dd, 6H, CH_3], δ 2.119 [m, 1H, CH], δ 3.059 [t, 2H, CH_2], δ 3.419 [d, 1H, CH] and δ 3.720 [t, 2H, CH_2]. The NMR spectrum was perfectly in accordance with the proposed structure.

Structures of HEVal and d_4-HEVal synthesized were identified by mass-data of GC–EI-MS (scan-mode) (Fig. 1). The molecular ion of HEVal (*m*/*z* 161) was not detected, but other

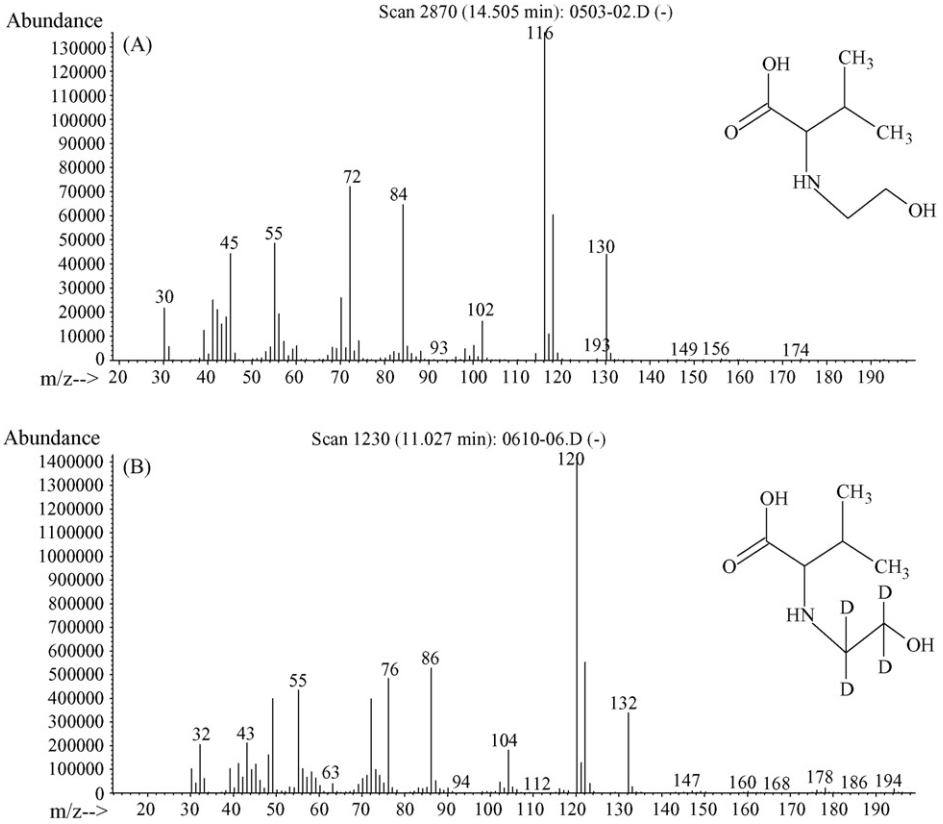

Fig. 1. EI spectra of (A) HEVal and (B) and d_4-HEVal synthesized.

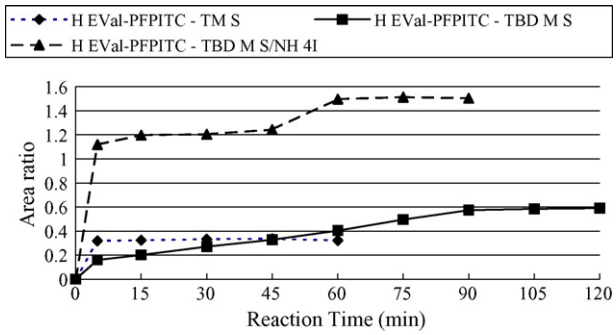

Fig. 2. Time courses of the reaction of PFPTH-HEVal with silylation reagents (MSTFA, MTBDMSTFA and MTBDMSTFA/NH_4-I (1000:4) containing 0.4 mg of dithioerythritol).

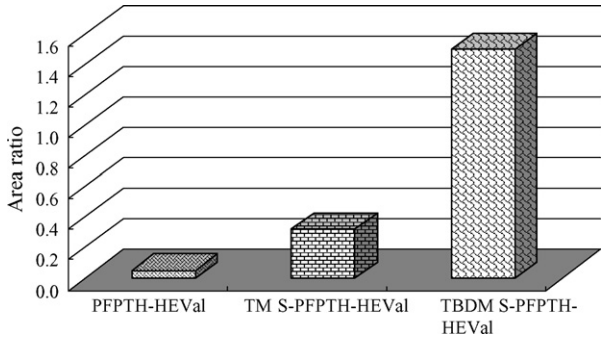

Fig. 3. Comparison of the sensitivity of PFPTH-HEVal and two derivatives by EI-MS (HEVal 171.5 pmol).

diagnostic ions at *m/z* 55, 72, 84, 100, 116, 118 and 130 of spectrum indicate HEVal. The ion at *m/z* 130 was from the loss of hydroxyl methyl group and the ion at *m/z* 116 from the loss of carboxylic acid group from the molecular ion. The molecular ion of d_4-HEVal (*m/z* 165) was not also detected, but diagnostic ions at *m/z* 55, 76, 86, 104, 120 and 132 of spectrum indicate d_4-HEVal. The ion at *m/z* 132 was due to the loss of d_2-hydroxyl methyl group and the ion at *m/z* 120 to the loss of carboxylic acid group from the molecular ion.

3.2. Silylation

For the enhancement of GC performance (e.g. peak symmetry, resolution and peak height) and sensitivity of PFPTH-HEVal, the silylation of the hydroxyl group of HEVal was selected. Silylated derivatives are formed by the displacement reaction of active proton as a nucleophilic attack of the more electronegative oxygen atom upon the silicon atom of MSTFA or MTBDMSTFA. Each active proton replaced by the TMS or TBDMS alkyl group adds the molecular weight and correspondingly the mass-to-charge ratio (*m/z*) of the analyte by 72 or 114, respectively. The silylating reagents, MSTFA (100%), MTBDMSTFA (100%) and MTBDMSTFA + 0.4% NH_4I containing 0.4 mg of dithioerthritol as catalyzing agent were compared to each other in terms of sensitivity, reactivity and interference from matrix. The derivatives were determined at intervals of 15 min until about 120 min.

The reaction rate of PFPTH-HEVal with MSTFA (100%) was determined by the detection of the area ratio of the product to *d*10-pyrene (Fig. 2). PFPTH-HEVal showed rapid reaction with 100% MSTFA (100%). TMS-PFPTH-HEVal was detected as the silylated product of PFPTH-HEVal with MSTFA and showed complete reaction in 30 min at 60 °C.

The reaction rates of PFPTH-HEVal with MTBDMSTFA (100%) and MTBDMSTFA-NH_4-I (1000:4, w/w) containing 0.4 mg of dithioerthritol were determined by the detection of the area ratio of TBDMS-PFPTH-HEVal to *d*10-pyrene. TBDMS-PFPTH-HEVal showed slow and incomplete reaction with MTBDMSTFA, but relatively rapid reaction with MTBDMSTFA-NH_4I (1000:4) containing 0.4 mg of dithioerthritol in about 60 min at 80 °C (Fig. 2). TBDMS-PFPTH-HEVal showed more sensitive and was not interfered from matrix than TMS-PFPTH-HEVal. As a result, MTBDMSTFA/NH_4I containing 0.4 mg of dithioerythritol was selected as silylation reagents of PFPTH-HEVal (Fig. 3).

3.3. Mass spectra of silylated derivatives

The mass spectra of the silylated derivatives by EI-MS are shown in Fig. 4. The molecular ion of TBDMS-PFPTH-HEVal (*m/z* 482) was not detected. Diagnostic ions at *m/z* 73, 101, 172, 383, 425 (base ion) and 467 of spectrum (A) indicated that PFPTH-HEVal was silylated to the corresponding TBDMS-PFPTH-HEVal with MTBDMSTFA. The ion at *m/z* 467 was from the loss of methyl group and the ion at *m/z* 425 from the loss of butyl group from the molecular ion.

The molecular ion of TBDMS-PFPTH-d_4-HEVal (*m/z* 486) was not also detected. Diagnostic ions at *m/z* 73, 104, 176, 387, 429 (base ion) and 471 of spectrum (B) indicated that PFPTH-d_4-HEVal (ISTD) was silylated to the corresponding TBDMS-PFPTH-d_4-HEVal with MTBDMSTFA. The ion at *m/z* 471 was due to the loss of methyl group and the ion at *m/z* 429 to the loss of carboxylic acid group from the molecular ion.

3.4. Chromatography

Fig. 5 shows the chromatograms of the TBDMS-PFPTH-HEVal and the TBDMS-PFPTH-d_4-HEVal (ISTD) following derivatization after the extraction from human blood. For the GC separation of the derivatives, the use of a non-polar stationary phase was found to be efficient. The peaks were symmetrical and no tailing could be seen. The derivatives also did not show any adsorption effect in the GC system. The retention times of ISTD and TBDMS-PFPTH-HEVal were 13.59 and 13.60 min, respectively. The separation of the derivatives from the background of globin was good.

3.5. Recovery

Five globin samples at the concentration of 311 pmol/g were prepared and the relative recovery was calculated by percentage of the derivative recovered. The recovery of HEVal was about 81.6% with the coefficient of variation of 4.6% (Table 1).

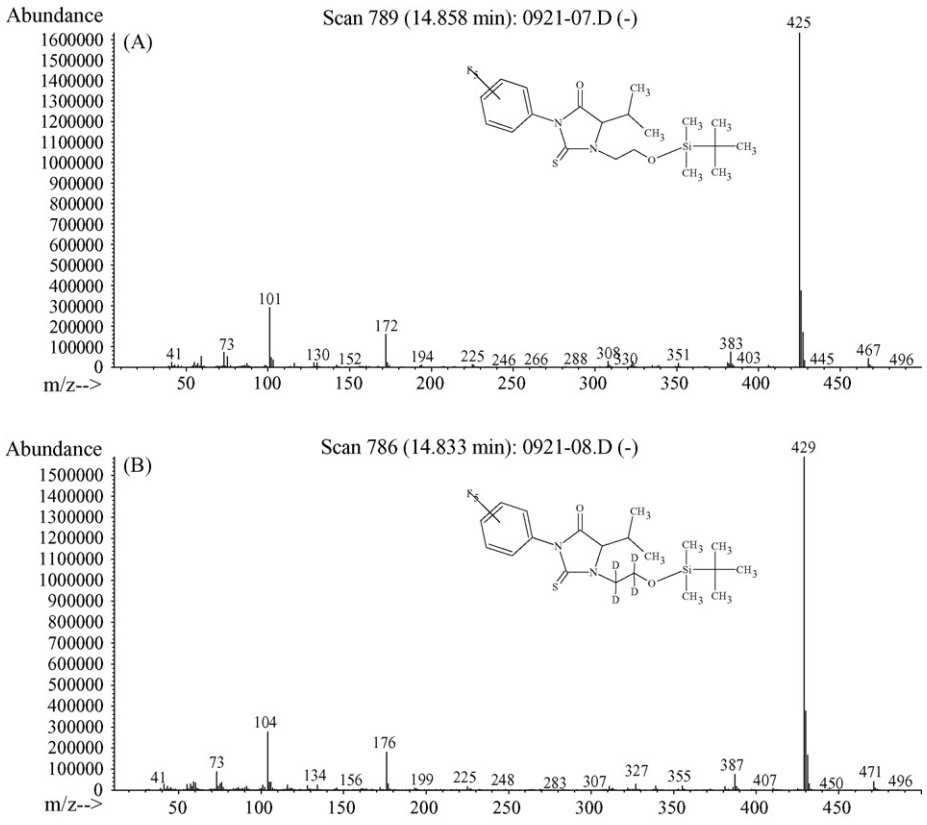

Fig. 4. EI mass spectra of (A) TBDMS-PFPTH-HEVal derivative and (B) TBDMS-PFPTH-d_4-HEVal derivative.

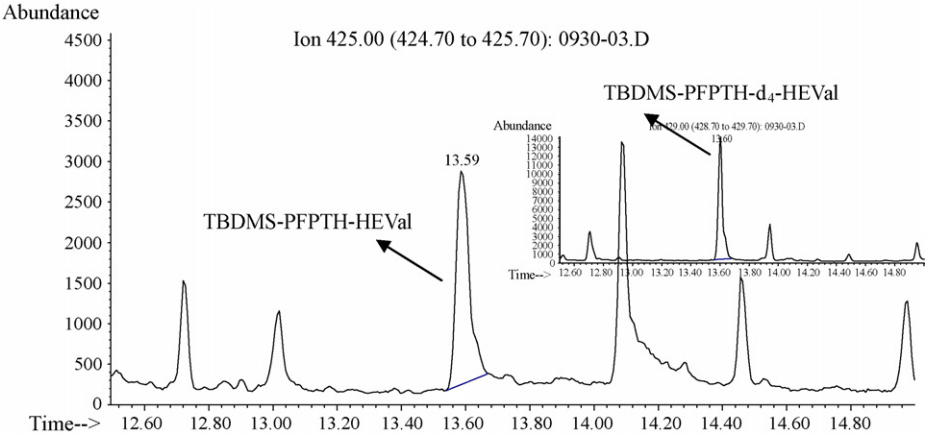

Fig. 5. GC–MS (SIM) chromatograms after the extraction and the derivatization of EO-adduct isolated from human blood (HEVal in figure was quantified in concentration of 27 pmol/g).

3.6. Linearity

Examination of typical standard curve by computing a regression line of peak area ratio of TBDMS-PFPTH-HEVal to ISTD on concentration using least-squares demonstrated a good fit with correlation coefficient being consistently greater than 0.9999. The line of best fit for HEVal is $y = 0.0214x + 0.0005$ ($r^2 = 0.9999$) over a range of 0.6–2484 pmol in 0.1 g of globin, where x is the analyte concentration (nmol/g) and y is the peak area ratio of the analyte to ISTD.

Table 1
Recovery result of HEVal in globin ($n = 5$)

Concentration (pmol/g)		X (%) ± S.D. (%)
Added	Found (%)	
310.5	84.8, 87.1, 76.0, 82.2, 78.0	81.6 ± 4.6

3.7. Low limit of detection

LOD was determined by the extraction of standard spiked in reagent blank and defined by three times of coefficients of vari-

Table 2
Comparison of analytical methods for determining HEVal in globin

Ref.	Sample amount (mg/Hb)	Preparation method	Instrument	LOD (pmol/g)
[16]	50	Edman-degradation	GC–EI-MS	9.0
[17]	50	Edman-degradation	GC–NICI-MS GC–EI-MS	1–10 20
This study	100	Edman-degradation + TBDMS silylation	GC–EI-MS	1.8

ation for replicate determinations ($n = 5$). LOD was 1.8 pmol/g for HEVal based 0.1 g of sample. The high sensitivity of the TBDMS-derivative by GC–EI-MS (SIM) permits the determination of HEVal at concentration well below than those reported in literatures previously without using of expensive instrument (Table 2).

3.8. Precision and accuracy

The reproducibility of the assay was very good. For five independent determinations of spiked samples spiked in control globin in the concentration of 311 pmol/g, mean 289 pmol/g was detected with accuracy of 93% and CV of 5.0% (Table 3).

3.9. Real sample analysis

As described in Table 2, the presented method presents more developed value in terms of LOD compared to the other methods.

We applied this method to determine the background concentrations of HEVal in human bloods of 20 residents in near of Ulsan-petroleum industrial complex in Korea. As a result, HEVal as hemoglobin adduct was detected in the concentration range of 12–6570 pmol/g in human blood samples (Fig. 6).

Table 3
Within-run precision and accuracy of HEVal in globin ($n = 5$)

Concentration (pmol/g)		$X \pm$ S.D. (R.S.D., %)
Added	Found	
310.5	268.5, 304.5, 292.0, 299.8, 282.0	289.4 ± 0.015 (5.0)

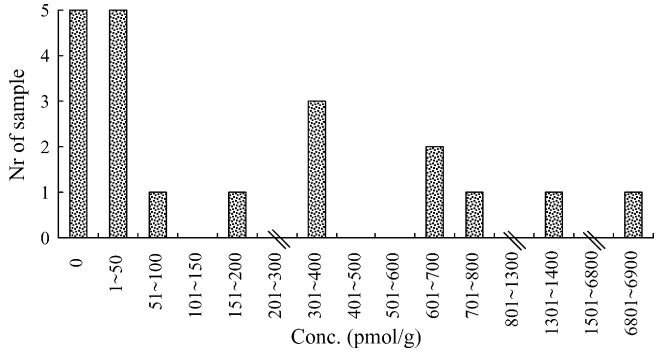

Fig. 6. The analytical results of human samples ($n = 20$).

4. Conclusions

The TBDMS derivative of PFPTH-HEVal with MTBDMSTFA/NH_4I (1000:4) containing dithioerythritol had good chromatographic property and offered very sensitive response of the GC–EI-MS (SIM). Quantification of HEVal was excellent, with linear calibration curves over a range of 0.6–2484 pmol in 0.1 g of globin and LOD was 1.8 pmol/g for HEVal.

The present method may be applicable to determine HEVal in human blood as biomarker for EO exposure by GC–EI-MS (SIM).

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