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Methyl vinyl ketone—Identification and quantification of adducts to N-terminal valine in human hemoglobin☆

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ABSTRACT

Adducts to N-terminal valines in Hb have been shown useful as biomarkers of exposure to electrophilic compounds. Adducts from many compounds have earlier been measured with a modified Edman degradation method using a GC–MS/MS method. A recently developed method, the adduct FI*R*E procedure™, adopted for analysis by LC–MS/MS, has been applied in this study. With this method a fluorescein isothiocyanate (FITC) reagent is used to measure adducts (*R*) from electrophiles with a modified Edman procedure. By using LC–MS/MS in product ion scan mode, a new peak was identified and the obtained MS data indicated that this adduct could originate from methyl vinyl ketone (MVK). Incubation of human-, sheep- and bovine blood with MVK increased the signal of the identified peak. By comparing the LC–MS/MS data from the unknown background peak with data obtained from synthesized fluorescein thiohydantoin (FTH) standards of the MVK adduct to valine and d_8-valine, the identity of this adduct was confirmed. The MVK adduct was shown present in human blood (~35 pmol/g globin, $n = 3$) and only just above LOD in bovine blood, $n = 1$ (LOD = 2 pmol/g globin). MVK reacts, in similarity with acrylamide, via Michael addition. MVK is known to occur in the environment and has earlier been observed in biological samples, which means that there are possible natural and anthropogenic exposure sources. Analysis of an Hb adduct from MVK in humans has to our knowledge not been described before.

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1. Introduction

The methods for analysis of hemoglobin (Hb) adducts, developed in the 1970–80s, primarily aimed to assess occupational exposures to reactive compounds/metabolites [1]. Along with improvement of the sensitivity and reliability of the methods, it was experienced that adducts from many of the studied compounds also were present in persons without known environmental exposure which was referred to as "background" adduct levels.

The N-alkyl Edman method for the detachment of adducts to N-terminal valine in Hb and analysis by GC–MS/MS has been used for studies of background adduct levels in non-smokers. One such example is the adduct formed from ethylene oxide [2,3], which was shown to mainly originate from endogenously formed ethylene/ethylene oxide [4]. Another example is the *N*-(2-carbamoylethyl)valine adduct, which was proven to originate from acrylamide, shown to be formed in food during cooking [5,6]. These findings illustrate the potential of protein adducts as a tool to identify exposures to electrophilically reactive compounds in the general population, originating from endogenous or exogenous sources.

The recently developed "adduct FI*R*E procedure™" for LC–MS/MS analysis of adducts to N-terminal proteins, is based on the same principle as the N-alkyl Edman method, which means that adducts to N-terminals are selectively detached and enriched (see Fig. 1). The abbreviation FI*R*E means that the fluorescein isothiocyanate (FITC) reagent is used to measure adducts (*R*) from electrophiles with a modified Edman procedure. The formed fluorescein thiohydantoin (FTH) analytes showed high sensitivity in the LC–MS/MS analysis [7–9]. This method opens for studies of a broader range of adducts as the LC–MS/MS technique is less discriminating than GC–MS/MS with regard to the adducts that can be measured. This means that the FI*R*E procedure has a potential to measure adducts that are charged, have high polarity, relatively high molecular weight and have thermo-labile properties [8, our unpublished work]. We have now initiated the exploration of the adduct FI*R*E procedure™ to identify unknown background exposures to electrophilic compounds.

Using the LC–MS/MS instrument in the precursor ion scan mode with known m/z ratios for the FTH derivative of N-substituted valines, indicated the presence of several unidentified N-terminal valine adducts in human blood samples processed with the FI*R*E

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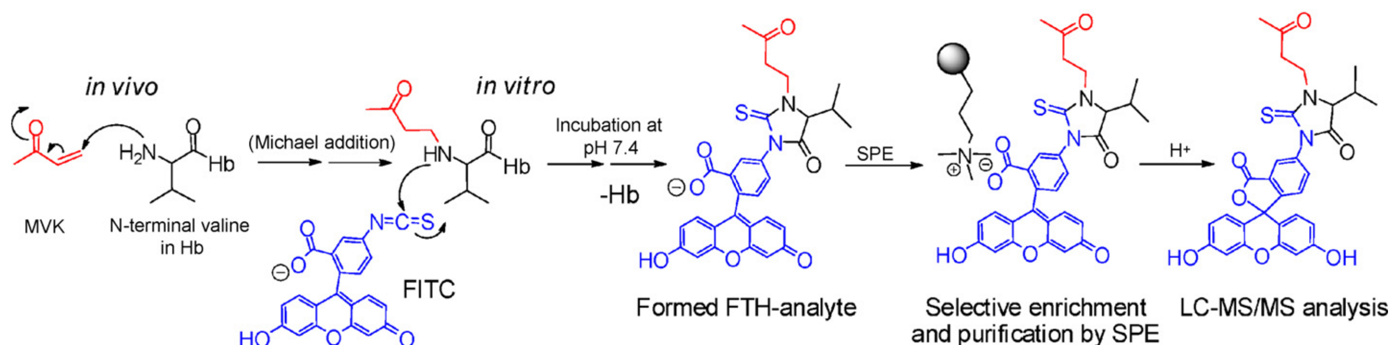

Fig. 1. Illustration of the formation of *N*-(butyl-3-one)valyl (MVK–Val) Hb adducts and measurement by the adduct FI*R*E procedure™.

procedure. The fragmentation pattern of one of the precursor ions had several similarities with the fragmentation pattern of the FTH analyte formed from the acrylamide adduct to valine. Our hypothesis, based on the MS/MS data, was that this peak could originate from methyl vinyl ketone (MVK). MVK was proposed since it, like acrylamide, is a conjugated type-2 alkene and could be expected to form adducts via a Michael addition reaction to N-terminals.

The aim of this study was to verify whether the observed unknown adduct to the N-termini in samples of human hemoglobin corresponds to the adduct formed from MVK.

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2. Material and methods

2.1. Chemicals

Caution: Methyl vinyl ketone (MVK), dioxan and fluorescein isothiocyanate (FITC) are hazardous and should be handled with care.

The reagent FITC that is fluorescein-5-isothiocyanate (isomer I, <90%) was from Fluka and was further purified as described below. MVK, L-valine (Val) and D,L-valine-d_8 (d_8-Val, 98% ^{2}H) and myoglobin (horse skeleton muscle) was obtained from Sigma–Aldrich. All other chemicals and solvents were of analytical grade. The analytical standards containing FTH analytes were stored in ACN/H_2O (1:1, v/v) at −20 °C until use. d_8-Valyl-leucyl anilide was synthesized according to Belov et al. [10]. This compound was incubated with MVK to form MVK–d_8-valyl-leucyl anilide as described below. Valine methylamide was synthesized according to Helleberg et al. [11].

FITC (2.0 g) was dissolved in ethyl acetate (EtOAc, 100 mL) and purified by extraction with water (3× 50 mL). In order to obtain homogenous phases ethanol was added in small portions when needed. The organic phase was dried by magnesium sulfate, the eluate filtered and then concentrated to around 1/10 of the original volume under vacuum on a rotary evaporator. The concentrated homogenous solution was purified on a silica gel column (30 mm × 250 mm) eluted with EtOAc. The first colored band was collected containing FITC with a purity of ca 95% (estimated by TLC). After evaporating to dryness, the obtained FITC (1.4 g) was grained and stored in vacuo with phosphorus pentoxide.

2.2. Synthesis of reference compounds

2.2.1. N-(Butyl-3-one)-L-valine methylamine (MVK–VMA)

MVK (78 mg, 1.1 mmol) and L-valine methylamide (VMA) (150 mg, 1.1 mmol) were dissolved in tetrahydrofuran (THF) (1 mL) and incubated for 3 days at 45 °C while following the reaction by TLC [methanol (MeOH)/acetonitrile (ACN)/EtOAc, 1:40:10, spot developed with $KMnO_4$, R_f = 0.4]. The solvent was evaporated and the obtained oil was directly derivatised with FITC as described below. The yield of MVK–VMA in the crude product was estimated to be between 40 and 60% (estimated by TLC).

2.2.2. Fluorescein-5-[4-isopropyl-3-(butyl-2-one)-2-thioxo-imidazolidin-5-one] (MVK–Val-FTH, 1)

The crude product of the MVK–VMA synthesis was dissolved in dioxan/H_2O (4:1) (2.5 mL) and reacted with FITC (50 mg, 0.13 mmol, 0.12 ekv to the synthesis of MVK–VMA) and alkalized with KOH (5.0 mg, 0.13 mmol). The solution was stirred for 3 h at 45 °C and the progress followed by TLC [dichloromethane (DCM)/EtOAc, 1:2, yellow spots, R_f = 0.3]. The solvent was evaporated and the product was diluted with 0.2 M aqueous cyanoacetic acid (CNHOAc) (30 mL) and extracted with EtOAc (2× 30 mL). The organic phase was dried with $MgSO_4$, and the eluate concentrated by rotary evaporation. The remaining oil was purified by a silica gel column eluted by EtOAc/DCM (1:1) (R_f = 0.5), to give 30 mg (54 μmol) of the desired product. Characterization was done by LC–MS/MS (see Table 1 and Fig. 2).

2.2.3. D,L-d_8-Valyl-leucyl anilide hydrochloride (d_8-ValLeuNHPh-HCl)

The synthesis was performed as described by Belov et al. [10] in 1.0 mmol scale to give white crystals with a m.p. of 215 °C. Analysis by LC–MS/MS (ESI, positive ion mode), product ion scan mode of m/z = 348 [M+1]$^+$, direct infusion at 10 μL/min using ACN:H_2O as mobile phase with the collision energy ramped between 10 and 50 V gave m/z (rel. ion intensity); 348.4 [(M+1)$^+$, 5%], 241.4 [(M+1−107)$^+$, 4%], 193.4 [(M+1−155)$^+$, 44%], 86.3 [(M+1−262)$^+$, 62%], 80.2 [(M+1−268)$^+$, 100%].

2.2.4. N-(Butyl-3-one)-D,L-d_8-valyl-leucyl anilide hydrochloride (MVK–d_8-ValLeuNHPh)

MVK (73 μL, 70 mg, 0.71 mmol), d_8-ValLeuNHPh-HCl (0.25 g, 0.71 mmol) and tertbutyl diisopropylamine (iPr_2NHtBu) (0.12 mL, 0.092 g, 0.71 mmol) were dissolved in EtOH (0.5 mL) and stirred at room temperature for 2 days. The reaction was followed by TLC [SiO_2, dichloromethane, spots developed with UV and $KMnO_4$: R_f = 0.5 (desired product), R_f = 0.6 (MVK), R_f = 0.1–0.3 (d_8-ValLeuNHPh), iPr_2NHtBu (base line)]. The solvent was evaporated and ACN (20 mL) was added. The precipitate (salt) was filtered off and the crude product was further purified on a silica gel column with EtOAc/DCM (2:1) mobile phase, fractionated and evaporated to yield 0.184 g (yield below 49%, 0.4 mmol, this product contained impurities) as an oil. Analysis by LC–MS/MS (ESI, positive ion mode), product ion scan mode of m/z = 384.4 [M+1]$^+$, direct infusion at 10 μL/min using ACN:H_2O as mobile phase with a collision energy of 20 V gave m/z (rel. ion intensity); 348.3 [(M+1)$^+$, 9%], 326.6 [(M+1−22)$^+$, 8%], 291.5 [(M+1−57)$^+$, 9%], 263.4 [(M+1−85)$^+$,

Table 1
LC–MS/MS (ESI) in the positive and negative ion mode by enhanced product ion scan of studied analytes. Figures presented in brackets are relative intensities.

Studied compounds (comp. nr)	Collision energy (V)	Molecule ion [M+H]+	P-I	P-II	P-III	P-IV	P-V	P-VI	P-VII	P-VIII	P-IX	P-X
MVK–Val-FTH (**1**)	50 (pos.)	559.1 (23)	516.3 (15)	501.1 (2)	489.2 (12)	487.2 (2)	473 (10)	460.3 (3)	445.1 (40)	417.1 (12)	402.4 (8)	390.1 (100)
MVK–d_7-Val-FTH (**2**)	50 (pos.)	566.2 (30)	516.3 (20)	501.1 (2)	496.4 (20)	487.4 (2)	473.1 (8)	461.3 (2)	445.2 (50)	417.1 (15)	409.4 (6)	390.2 (100)
		Molecule ion [M-H]$^-$	P-I	P-II	P-III	P-IV	P-V	P-VI	P-VII	P-VIII	P-IX	P-X
MVK–Val-FTH (**1**)	−40 (neg.)	557.2 (60)	513.2 (100)	512.2 (95)	487.1 (3)	485.1 (3)	472.3 (4)	469.1 (4)	443.0 (33)	441.1 (20)	401.4 (25)	382 (18)
MVK–d_7-Val-FTH (**2**)	−40 (neg.)	564.3 (40)	520.2 (80)	519.2 (100)	494.5 (5)	492.3 (3)	479.2 (2)	476.2 (2)	450.4 (30)	448.1 (20)	408.0 (10)	389.4 (15)

17%], 233.4 [$(M+1-115)^+$, 39%], 205.5 [$(M+1-143)^+$, 42%], 177.4 [$(M+1-171)^+$, 35%], 150.3 [$(M+1-198)^+$, 100%].

2.2.5. Fluorescein-5-[4-d_7-isopropyl-3-(butyl-2-one)-2-thioxo-imidazolidin-5-one] (MVK–d_7-Val-FTH, 2)

MVK–d_8-ValLeuNHPh (150 mg, 0.43 mmol) and $KHCO_3$ (43 mg, 0.43 mmol) was dissolved in dioxan/H_2O (4:1) (5 mL). FITC (50 mg, 0.14 mmol) was added and the reaction was stirred at 55 °C for 2 h. The solvent was concentrated by rotary evaporation to a volume of 0.5 mL, diluted with 0.2 M aqueous CNHOAc (30 mL), extracted and purified as described above (see MVK–Val-FTH) to yield 40 mg of a crude product. One aliquot of the crude product (10 mg) was further purified by HPLC using a C_{18} column to give 3 mg (5 μmol of the desired product; <95% purity determined by LC–MS/MS). Characterization was done by LC–MS/MS (see Table 1 and Fig. 2).

2.3. Blood samples

Human blood was purchased from Karolinska University Hospital (Stockholm, Sweden), the donors were non-smokers, no further information regarding, sex, age etc. was obtained. Defibrinated sheep blood and bovine blood (with citrate) were purchased from the National Veterinary Institute (Uppsala, Sweden).

The blood was treated in two different ways: (a) Whole blood was placed in freezer at −20 °C to achieve hemolysis. (b) The blood was separated into red blood cells and plasma by centrifugation (10 min, 4500 g) and the plasma and buffy coat was removed before freezing. The Hb content was measured with a HemoCue instrument and the Hb content was adjusted with water to be approximately 140 g/L. This blood was then processed as described in Section 2.7.

2.3.1. Incubation of blood samples

Lysed whole blood (1 mL) was incubated with MVK (0.25 mmol, 20 μL) to give a concentration of 0.25 M MVK. The reaction was performed at 37 °C for 1 h and the analysis of the formed adducts to N-terminal valine in Hb were done from aliquots (250 μL, $n = 2$) by the adduct FIRE procedure, see Section 2.7.

2.4. Equipment for sample preparation

The solid phase extraction (SPE) cartridges Oasis Max (60 mg) were obtained from Waters (Milford, MA, USA). A thermomixer comfort and a 5804 R centrifuge with rotor A-4-44 and F-45-30-11 (Eppendorf Nordic, Denmark) were used for preparation of blood samples derivatised with FITC. The Hb analyzer (Hb 201+) was obtained from HemoCue (Ängelholm, Sweden).

2.5. Liquid chromatography–mass spectrometry

The LC–MS/MS system consisted of a Shimadzu Prominence LC 20 system (Shimadzu Corp., Kyoto, Japan) interfaced to an API 3200 Q-trap instrument with a turbo TurboIonSpray® interface (ESI), Applied Biosystems/MDS Sciex (Concord, ON, Canada). A Fortis C_{18} column was used (2.1 μm, 2.1 mm × 150 mm) (Fortis Technologies Ltd., Cheshire, United Kingdom). The mobile phase consisted of, A: 0.1% formic acid in H_2O/ACN (95:5, v/v), and B: 0.1% formic acid in H_2O/ACN (5:95, v/v). A gradient was applied from 30% B to 50% B in 10 min, and then stepped to 100% B in 5 min and was kept for 5 min before re-equilibrating the column with the initial mobile phase. The injection volume was 20 μL and the flow rate was 125 μL/min. Instrument settings for the mass spectrometer with the offset values in V: declustering potential 80 V, entrance potential 10 V, collision energy 56 V, nebulizer gas (N_2) 30 (arbitrary units, au), turbo gas (N_2) 20 au, curtain gas (N_2) 30 au, collision

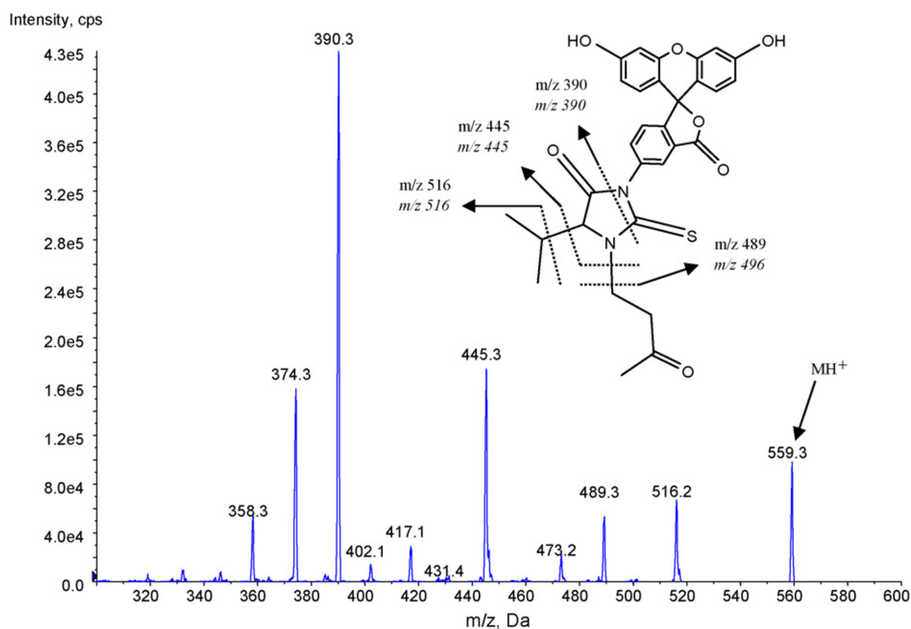

Fig. 2. Product ion spectra (ESI, positive ion mode, CE = 56) and proposed fragmentation schemes of MVK–Val-FTH (m/z = 559). m/z ratios written in italics correspond to the deuterated derivative MVK–d_7-Val-FTH (spectrum not shown).

gas (N_2) 5 au, ion spray voltage 4500 V and vaporizing temperature 400 °C. In product ion mode a varied collision energy was applied. Analysis of processed samples was performed in the positive ion mode, using multiple reaction monitoring (MRM) with the following transitions: MVK–Val-FTH m/z 559.1 → 516.1, MVK–d_7-Val-FTH m/z 566.1 → 516.1. Fragments presented in Table 1 were obtained with the linear ion trap function (enhanced product ion scan).

For the comparison of MS/MS spectra, the fragmentation of the following compounds was used: Val-FTH, Me-Val-FTH, glycidamide-Val-FTH (GA-Val-FTH), d_3-GA-Val-FTH, GA-d_7-Val-FTH, acrylamide-Val-FTH (AA-Val-FTH), d_3-AA-Val-FTH, AA-d_7-Val-FTH, ethylene oxide-Val-FTH (EO-Val-FTH) and EO-d_7-Val-FTH.

2.6. Calibration curve

Quantification was conducted using internal standard calibration with the hepta-deuterated FTH analyte (MVK–d_7-Val-FTH). The calibration curve was established as the area ratios between analyte and internal standard versus added amount of analyte per sample. The calibration samples were prepared by adding the standards, diluted in ACN/H_2O 3:7, as follows: 0.28, 0.56, 1.13, 2.25, 4.5, 9.0 and 18.0 pmol to 250 μL of sheep blood with Hb 130 g/L. The samples were then processed as described in section 2.7. The added amount of internal standard was 5 pmol/250 μL of sample. The limit of quantification (LOQ) was set to 10 times the noise and limit of detection (LOD) was set to 3 times the noise.

2.7. Analysis of blood samples by the FIRE procedure

General procedure: Isolated red blood cells (250 μL) prepared as described above (2.3) was transferred to Eppendorf tubes (1.5 mL) and alkalized with 1 M $KHCO_3$ (15 μL). FITC (5 mg, 13 μmol) dissolved in DMF (30 μL) was added and the samples were heated and mixed (37 °C at 800 rpm) on a thermomixer comfort for 8 h. The internal standards, MVK–d_7-Val-FTH and EO-d_7-Val-FTH in H_2O/ACN (50 μL, 6:4, v/v) were added from a concentration of 0.1 pmol/μL (5 pmol of IS/sample). The precipitation of proteins was performed by adding ACN (1.6 mL) under mixing, followed by centrifugation (10 min at 15 500 g). The pH was adjusted by addition of 1 M ammonium hydroxide (25 μL) to the supernatant before it was transferred to SPE mixed-mode anion exchange cartridges (Oasis MAX). A washing procedure with ACN, H_2O and 0.5% aqueous cyanoacetic acid (2 mL of each solvent) was performed and the analytes were eluted with 0.25% cyanoacetic acid in H_2O/ACN (1.4 mL, 4:6, v/v). The solvent was evaporated to dryness under a gentle stream of air and the solid residue was dissolved in H_2O/dioxan (80 μL, 7:3, v/v) prior to analysis by LC–MS/MS.

3. Results

3.1. Identification of MVK–Hb adducts

In human blood samples processed according to the adduct FIRE procedure, and analyzed by LC–MS/MS (ESI, positive ion mode) in the precursor ion mode, the M+1 ion with a m/z ratio of 559 were found. The fragments with m/z = 445 and 489 were used for the precursor ion scans, as they are common fragments for adduct-containing Val-FTH's. The fragmentation pattern of the M+1 ion had several m/z fragment ions that were commonly found for other studied Val-FTH analytes. The peak elutes at 17.6 min, after the *N*-(2-hydroxyethyl)valine adduct (EO-Val-FTH, 16.5 min) on the reversed phase (C_{18}) HPLC column. After comparison of fragmentation spectra and elution order, it was hypothesized that the analyte corresponded to the reaction product of MVK.

To confirm the identity of the observed adduct the same blood was incubated with MVK (0.25 M, 1 h, 37 °C). The analysis by the FIRE procedure using MVK–d_7-Val-FTH as IS showed that the intensity of the unknown peak in the chromatogram increased significantly and the retention time and fragmentation pattern remained unchanged. This adduct was not detected when myoglobin was used as a matrix in sample preparation instead of blood [40 mg dissolved in aqueous 0.5 M $KHCO_3$:1-propanol (1 mL, 2:1, v/v)]. The peak increased from a very low background adduct level to a peak with high intensity when sheep or bovine blood was incubated with MVK. The unknown background peak obtained in the LC–MS/MS (ESI, positive ion mode) analysis was compared with the synthesized reference compounds, MVK–Val-FTH and the IS,

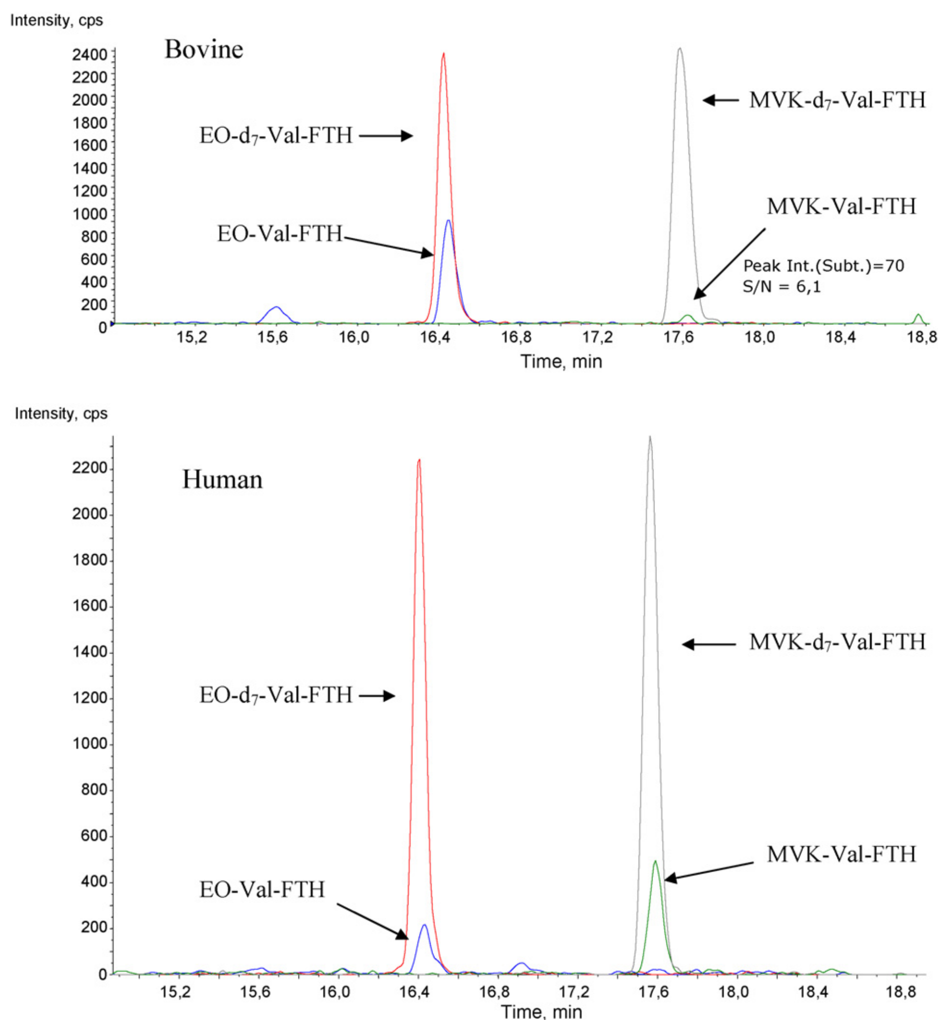

Fig. 3. Chromatogram from analysis of bovine and human blood samples of MVK–Val-FTH with MVK–d_7-Val-FTH as IS and as a comparison the adduct from ethylene oxide (EO-Val-FTH).

MVK–d_7-Val-FTH. The retention time and fragmentation pattern confirmed the identity of this adduct. The relative intensities of the obtained product ions of the peak obtained from MVK-incubated blood and of the peak from the reference standard MVK–Val-FTH were identical. Fragments obtained by enhanced product ion scan (MS/MS) in positive and negative ion mode are given in Table 1. MS/MS spectrum of MVK–Val-FTH is presented in Fig. 2 with interpretation of a few positive fragment ions.

3.2. *Analysis of MVK–Hb adducts in blood*

The N-terminal Hb adducts formed from MVK were present in all human blood samples processed ($n = 3$, non-smokers). The adduct levels of MVK were measured to be approx. 30, 32 and 40 pmol/g Hb. The MVK adduct was just above LOD in bovine blood ($n = 1$). A column with 2.1 μm particle size and a relatively slow gradient had to be applied to obtain a peak above LOD (2 pmol/g globin). The adduct level in the bovine sample was roughly estimated to 4 pmol/g globin, see Fig. 3. LOQ was estimated to 6 pmol/g Hb. The analytical precision was acceptable with a RSD of 5.2% ($n = 3$). Repeated processing of samples gave variation in the obtained adduct levels. The inter day precision ($n = 3$ days) varied between 18 and 28% for repeated analysis of the erythrocytes from the three individuals.

Information was obtained from the blood central that the donors were non-smokers. This information was also verified by simultaneous measurements of the adducts formed from ethylene oxide (EO-Val), which in smokers is significantly increased. The about one order of magnitude lower adduct level in bovine Hb as compared to human Hb could partly reflect the fact that bovine Hb just have two N-terminal valine instead of four as in human Hb.

4. Discussion

An observed unknown adduct to N-terminal valine in human Hb was confirmed to originate from MVK. Analysis of Hb adducts of MVK in humans has to our knowledge not been described before. MVK as such has though been found in biological samples as a volatile compound in urine from several animal species [12–14], and in human breath [15] and in the volatile phase from human faeces [16], thus indicating endogenous sources.

With regard to possible environmental sources it is known that MVK is used in the chemical industry, for instance for the production of plastics and of vitamin A [17]; MVK is formed in combustion of biomass [18,19], and is found in automobile exhaust [20] and in cigarette smoke [21]. The compound is formed in photooxidation of isoprene [22], and occurs in emissions from airborne dust from damp residences [23] and in moldy building materials [24]. MVK thus is present as a ubiquitous air pollutant [25,26]. Furthermore, MVK also occurs in the volatile phase from different foods [27–29]. This means that there are several known sources, anthropogenic as well as natural, for exposure to MVK.

Some low-molecular adducts might be formed from several electrophiles. However, the published data regarding MVK, showing that the compound occurs in biological samples and in the environment, strengthen that MVK is the origin of the observed adduct. A later question is whether this list of sources is complete and whether there is one major source of the MVK observed as an adduct to Hb.

Literature data show that MVK is a severely toxic compound and is highly irritating to mucous membranes and has show systemic toxicity [17,30–32]. *In vitro* test on genotoxicity do not permit any clear conclusions (see for instance Ref. [33]) and there are no data available from cancer tests [32]. MVK's ability to form adducts to nucleophilic sites in proteins *in vitro* has been described by others [34]. The α,β-unsaturated ketone possess electrophilic reactivity and DNA adduct formation has been verified *in vitro* [35,36], which suggests that the compound is genotoxic. The electrophilic reactivity of MVK was higher than acrylamide, but lower than acrolein when the second order rate constant was determined for the reaction with the sulfhydryl group of N-acetyl-cysteine [37].

In this work the presence of an adduct from MVK to N-terminal valine in human Hb from non-smokers was demonstrated and the identity of the adduct confirmed. For the evaluation of validation parameters for quantitative analysis, as well as the reactivity of MVK towards N-terminal valine in Hb, and the stability of the MVK–Hb adduct, further studies have been initiated.

Acknowledgements

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