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A new modified Edman procedure for analysis of N-terminal valine adducts in hemoglobin by LC–MS/MS☆

Hans von Stedingk[1], Per Rydberg*,[1], Margareta Törnqvist

Department of Materials and Environmental Chemistry, Arrhenius Laboratory, Stockholm University, SE-106 91 Stockholm, Sweden

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ABSTRACT

A rapid and sensitive method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for simultaneous determination of adducts from acrylamide, glycidamide and ethylene oxide to N-terminal valines in hemoglobin (Hb) was developed. This new procedure is based on the same principles as the N-alkyl Edman procedure for analysis of adducts from electrophilic agents to N-terminal valines in Hb. The N-substituted valines can be detached, enriched and measured selectively as thiohydantoins by the use of an Edman reagent, in this case fluorescein isothiocyanate (FITC). This procedure is denoted as the "adduct FIRE procedure" as the FITC reagent is used for measurement of adducts (R) formed from electrophilic compounds with a modified Edman procedure. In this study, fluorescein thiohydantoin (FTH) analytes of N-substituted valines from acrylamide, glycidamide and ethylene oxide, as well as their corresponding hepta- and tri-deuterium-substituted analogues, were synthesized. These analytes ($n = 8$) were then characterized by LC–MS/MS (ESI, positive ion mode) and obtained product ions were interpreted. A considerable work with optimization of the FIRE procedure™, resulted in a procedure in which low background levels of the studied adducts could be measured from 250 μL lyzed whole blood samples (human non-smokers). The analytes were enriched and purified with solid phase extraction columns and analyzed by LC–MS/MS with LOQ down to 1 pmol adduct/g Hb. Compared to other procedures for determination of N-terminal Hb adducts, the introduction of FITC has led to a simplified procedure, where whole blood also can be used, giving new opportunities and reduced hand on time with increased sample throughput.

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1. Introduction

The modified Edman procedure for analysis of adducts to N-termini in hemoglobin (Hb) was introduced in 1986 [1]. This procedure, the so called N-alkyl Edman method, has been applied to a range of compounds and in several respects was a break-through for the in vivo monitoring of simple alkylating agents as their Hb adducts (see for instance [1–6]). The method involves coupling of pentafluorophenyl isothiocyanate (PFPITC) to N-terminal valines in isolated globin, which leads to detachment, through cyclization, of the valines that are N-alkylated. The branched R group in the side-chain together with a N-substitution favours the cyclization of the thiocarbamoylated valine, through a gem-dialkyl effect [7]. The thiohydantoin formed in one step could then be isolated by extraction and analyzed with high sensitivity by GC–MS with negative ion chemical ionization (NICI).

The GC–MS technique has its limitations with regard to the analysis of thermo labile, hydrophilic and non-volatile analytes, which could be overcome with LC–MS techniques. Recently modifications of the N-alkyl Edman method for analysis by LC–MS have been published, using PFPITC [8–11] or the original Edman reagent, phenyl isothiocyanate (PITC) [12–15], as the reagent for detachment.

With the aim to develop a high-throughput method with a broad application range for analysis of Hb adducts we evaluated Edman reagents suitable for LC–MS analysis of the thiohydantoin derivatives. In our earlier work we compared three candidate Edman reagents with the previously used PITC and PFPITC reagents and found that fluorescein isothiocyanate (FITC) was the most suitable [16,17]. This study initiated the development of the "adduct FIRE procedure™", this means that the FITC reagent is used for the measurement of adducts (R) from electrophilic compounds with a modified Edman procedure (see Fig. 1). One criteria to achieve a high-throughput method was that the adducts should be isolated directly from blood, without prior isolation of globin.

This report summarizes the most critical parameters and their effect on the yields when processing blood samples for the analysis of background adduct levels. As model compounds the adducts

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* Corresponding author. Tel.: +46 8 163 993; fax: +46 8 163 979.
E-mail addresses: hans.stedingk@mmk.su.se (H. von Stedingk), per.rydberg@mmk.su.se (P. Rydberg), margareta.tornqvist@mmk.su.se (M. Törnqvist).

[1] H. von Stedingk and P. Rydberg contributed equally to this work.

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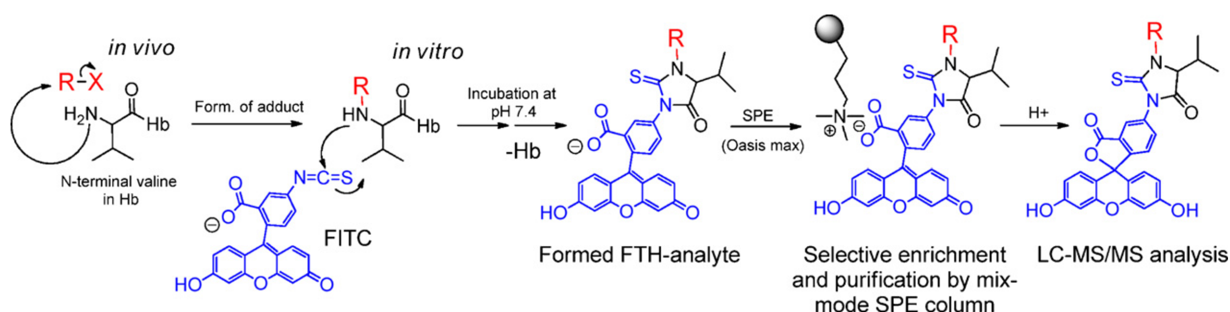

Fig. 1. Illustration of the adduct FIRE procedure.

Table 1
Studied analytes.

Structures	Analyte (comp. nr.)	Adduct (R_1)	isPr (R_2)
FTH analytes	AA-Val-FTH (**1**)	$-CH_2CH_2CONH_2$	Normal
	d_3-AA-Val-FTH (**2**)	$-CD_2CDHCONH_2$	Normal
	AA-d_7-Val-FTH (**3**)	$-CH_2CH_2CONH_2$	d_7
	GA-Val-FTH (**4**)	$-CH_2CH(OH)CONH_2$	Normal
	d_3-GA-Val-FTH (**5**)	$-CD_2CD(OH)CONH_2$	Normal
Normal analytes: $R_2 = CH(CH_3)_2$	GA-d_7-Val-FTH (**6**)	$-CH_2CH(OH)CONH_2$	d_7
	EO-Val-FTH (**7**)	$-CH_2CH_2OH$	Normal
Internal standards: $R_2 = CD(CD_3)_2$	EO-d_7-Val-FTH (**8**)	$-CH_2CH_2OH$	d_7

to valine from ethylene oxide, acrylamide and its metabolite glycidamide were used. These adducts are present in blood from non-smokers, due to general exposure to acrylamide via food [18] and to ethylene oxide from endogenous formation [6,19]. In the evaluation of the new adduct FIRE procedure™,[2] the background adduct levels from these compounds in human blood, at levels from about 15 pmol/g Hb were studied.

To our knowledge no procedure has so far been described for determination of N-terminal hemoglobin adducts starting from whole blood.

2. Material and methods

2.1. Chemicals and materials

Caution: Acrylamide, 2-bromoethanol, dioxan, glycidamide and isothiocyanate reagents are hazardous and should be handled with care.

The structures of compounds **1**–**10** and their abbreviated names are given in Table 1. Fluoresceine-5-isothiocyanate (isomer I, <90%), obtained from Fluka was purified before use as described below; 2-bromoethanol (Acros); acrylamide, ninhydrin (97%), L-valine (Val), and D,L-($^2H_8$)valine (d_8-Val, 98% ^2H) (Sigma–Aldrich), acrylamide (2,3,3-$^2H_3$, 98% ^2H), (CIL, Andover, MA) were used. Fluorescein-5-(4-isopropyl-2-thioxo-imidazolidin-5-one) (**1**, FTH-Val) and fluorescein-5-(4-isopropyl-3-methyl-2-thioxo-imidazolidin-5-one) (**2**, FTH-MeVal) were synthesized as described earlier [17]. All other chemicals and solvents were of analytical grade. All analytical standards containing FTH analytes were stored in acetonitrile (ACN):H_2O (1:1) at −18 °C until use.

[2] The TradeMark belongs to Adduct Analys AB, Skolvägen 18, Enebyberg, Sweden.

N-(2-Hydroxyethyl)-D,L-valine (EO-Val) was synthesized from α-bromoisovaleric acid and 2-amionethanol as described earlier [20]. Glycidamide and glycidamide-d_3 were synthesized by alkaline oxidation of acrylonitrile and acrylonitrile-d_3, respectively, with hydrogen peroxide anion as described earlier [21].

TLC was performed using silica gel 60 f-254 plates (SiO_2, Merck) and the spots were visualized with both UV (254 nm) and at long wavelength (378 nm). The response function (Rf–TLC) were obtained from toluene (Tol):ethyl acetate (EtOAc):ethanol (EtOH) (3:3:1, v/v/v) unless other stated.

2.2. Preparation of stock solutions

2.2.1. 1.0 M sodium valinate (Val-Na) stock solution

L-Valine (1.17 mg, 10 mmol) and NaOH (0.40 g, 10 mmol) was added to a test tube (10 mL), diluted with H_2O (8 mL), thoroughly mixed and then adjusted with H_2O to a final volume of 10 mL.

2.2.2. 1.0 M sodium d_8-valinate (d_8-Na) stock solution

L-d_8-Valine (125 mg, 1.0 mmol) and NaOH (40 mg, 1.0 mmol) was added to an Eppendorf tube (1.5 mL), diluted with H_2O (approx. 0.8 mL), thoroughly mixed and then adjusted with H_2O to a final volume of 1.0 mL. These stock solutions were stored at −18 °C.

2.3. Purification of FITC

FITC (2.0 g) was dissolved in EtOAc (100 mL) and purified by extraction with water (3 × 50 ml). In order to obtain homogenous phases EtOH was added in small portions when needed. The organic phase was dried by magnesium sulphate, 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 × 250 mm) eluted with EtOAc. The first coloured band was collected containing FITC with a purity of ca 95% (estimated by TLC). After evaporation to

dryness, the obtained FITC (1.4 g) was fine-grained and stored in vacuo with phosphorus pentoxide.

2.4. Syntheses of reference compounds

2.4.1. Sodium N-(2-carbamoylethyl)-L-valinate (AA-Val-Na)

General procedure: To an Eppendorf tube was 1 M Val-Na (1.0 mL, 1.0 mmol, see Section 2.2.1) and acrylamide (85 mg, 1.2 mmol) added. The mixture was heated and mixed on a thermomixer comfort for 18 h at 60 °C. The disappearance of Val-Na was monitored by TLC (SiO_2, plates eluted with chloroform, methanol and 17% aqueous ammonia, 2:2:1), spots developed with ninhydrin (Rf = 0.65). This reaction mixture, as well as the below described syntheses of N-substituted valinates, were derivatised directly with FITC as described below (Sections 2.4.8–2.4.15).

2.4.2. Sodium N-(2-carbamoyl-1,1,2-d_3-ethyl)-L-valinate (d_3-AA-Val-Na)

1.0 M Val-Na (0.20 mL, 0.20 mmol, see Section 2.2.1) prepared as described above was incubated with acrylamide-d_3 (18 mg, 0.24 mmol, 1.2 equiv.) in a 0.5 mL tube (Eppendorf) for 3 days at 37 °C. The reaction was monitored as described above.

2.4.3. Sodium N-(2-carbamoylethyl)-L-d_8-valinate (AA-d_8-Val-Na)

1.0 M d_8-Val-Na (0.30 mL, 0.30 mmol, see Section 2.2.2) was incubated with acrylamide (25 mg, 0.35 mmol, 1.2 equiv.) in a 0.5 mL tube (Eppendorf) for 3 days at 37 °C.

2.4.4. Sodium N-(2-carbamoyl-2-hydroxy ethyl)-L-valinate (GA-Val-Na)

1.0 M Val-Na (1.0 mL, 1.0 mmol, see Section 2.2.1) was incubated with glycidamide (102 mg, 1.2 mmol, 1.2 equiv.) in a 1.5 mL tube (Eppendorf) for 18 h at 50 °C.

2.4.5. Sodium N-(2-carbamoyl-2-hydroxy-1,1,2-d_3-ethyl)-L-valinate (d_3-GA-Val-Na)

1.0 M Val-Na (0.50 mL, 0.50 mmol, see Section 2.2.1) was incubated with glycidamide-d_3 (53 mg, 0.60 mmol, 1.2 equiv.) in a 1.5 mL tube (Eppendorf) for 18 h at 50 °C.

2.4.6. Sodium N-(2-carbamoyl-2-hydroxyethyl)-L-d_8-valinate-Na (GA-d_8-Val-Na)

1.0 M d_8-Val-Na (0.30 mL, 0.30 mmol, see Section 2.2.2) was incubated with glycidamide (30 mg, 0.35 mmol, 1.2 equiv.) in a 0.5 mL tube (Eppendorf) for 18 h at 50 °C.

2.4.7. Sodium N-(2-hydroxyethyl)-L-d_8-valinate (EO-d_8-Val-Na)

1.0 M d_8-Val-Na (0.30 mL, 0.30 mmol, see Section 2.2.2) was diluted with H_2O (0.3 mL) and ethanol (0.3 mL) in a 1.5 mL tube (Eppendorf) and incubated with 2-bromoethanol (25 μL, 0.35 mmol, 1.2 equiv.) for 3 days at 60 °C. In order to neutralize released HBr two portions of 1 M NaOH (175 μL, 0.175 mmol) was added after 24 and 48 h.

2.4.8. Fluorescein-5-[4-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one] (**1**, AA-Val-FTH)

General procedure: The whole reaction mixture of the incubated AA-Val-Na solution (1.0 mmol, see Section 2.4.1) was diluted with 0.125 M potassium hydrogen carbonate ($KHCO_3$) (3 mL) and mixed with a 2 mL dioxan solution of FITC (195 mg, 0.50 mmol) The reaction mixture was heated at 45 °C under magnetic stirring. After 90 min all FITC was consumed and a new spot was formed as monitored by TLC (aliquots were mixed with 2% cyanoacetic acid in ethanol in 1:5 ratio, plates eluted with toluene:EtOAc:EtOH; 3:3:1, v:v, Rf–TLC = 0.65 for FITC, Rf = 0.39 for **1**). The reaction solution was acidified with 1 M HCl (2 mL, 2 mmol) and extracted with H_2O:EtOAc (15 + 15 mL), the organic phase was washed with two additional volumes of H_2O, then dried with $MgSO_4$, and the filtrate was evaporated to dryness under vacuo. The product was purified by column chromatography (SiO_2, 20 × 200 mm) eluted with Tol:EtOH (3:3:1, v/v) and isolated in fractions which were combined and dried in vacuo to yield 205 mg (0.37 mmol, 74%) of the desired product as a solid compound (pure according to TLC, Rf = 0.39 and LC–MS). LC–MS/MS analysis, see Table 2 and Fig. 2.

2.4.9. Fluorescein-5-[4-isopropyl-3-(2-carbamoyl-1,1,2-d_3-ethyl)-2-thioxo-imidazolidin-5-one] (**2**, d_3-AA-Val-FTH)

The d_3-AA-Val-Na solution (0.20 mmol, see Section 2.4.2) was diluted with 0.125 M $KHCO_3$ (1 mL) and mixed with a 0.5 mL dioxan solution containing FITC (58 mg, 0.15 mmol). The synthesis was performed as described above but purification of the product was performed in a five times smaller scale. After purification on silica gel column the desired product was obtained to yield 60 mg (0.11 mmol, 71%, pure according to TLC, Rf = 0.39, and LC–MS) as a solid compound. LC–MS/MS analysis, see Table 2 and Fig. 2.

2.4.10. Fluorescein-5-[4-d_7-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one] (**3**, AA-d_7-Val-FTH)

The AA-d_8-Val-Na solution (0.30 mmol, see Section 2.4.3) was diluted with 0.125 M $KHCO_3$ (2 mL) and mixed with a 1.0 mL dioxan

Table 2
LC–MS/MS (ESI+) product ion scan of studied analytes (comp. **1**–**8**). Figures presented in brackets are relative intensities. Proposed structures of ions P-I to P-VIII are given in Fig. 2.

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
AA-Val-FTH (**1**)	50	560 (100)	517 (52)	–	489 (26)	–	–	445 (37)	390 (81)	374 (52)
d_3-AA-Val-FTH (**2**)	54	563 (40)	520 (36)	–	489 (30)	–	–	445 (46)	390 (100)	374 (50)
AA-d_7-Val-FTH (**3**)	50	567 (100)	517 (38)	–	496 (30)	–	–	445 (28)	390 (63)	374 (36)
GA-Val-FTH (**4**)	50	576 (100)	533 (7)	531 (55)	489[a] (30)	489[a] (30)	460 (28)	445 (16)	390 (90)	374 (17)
d_3-GA-Val-FTH (**5**)	54	579 (60)	536 (15)	534 (100)	489 (20)	492 (70)	462 (74)	445 (25)	390 (63)	374 (20)
GA-d_7-Val-FTH (**6**)	50	583 (100)	533 (8)	538 (52)	496 (18)	489 (25)	460 (23)	445 (18)	390 (59)	374 (26)
EO-Val-FTH (**7**)	50	533 (95)	490 (27)	–	489 (8)		460 (23)	445 (32)	390 (80)	374 (100)
EO-d_7-Val-FTH (**8**)	50	540 (100)	490 (28)	–	496 (4)		460 (23)	445 (25)	390 (68)	374 (73)

[a] P-II and P-III give rise to the same *m/z*.

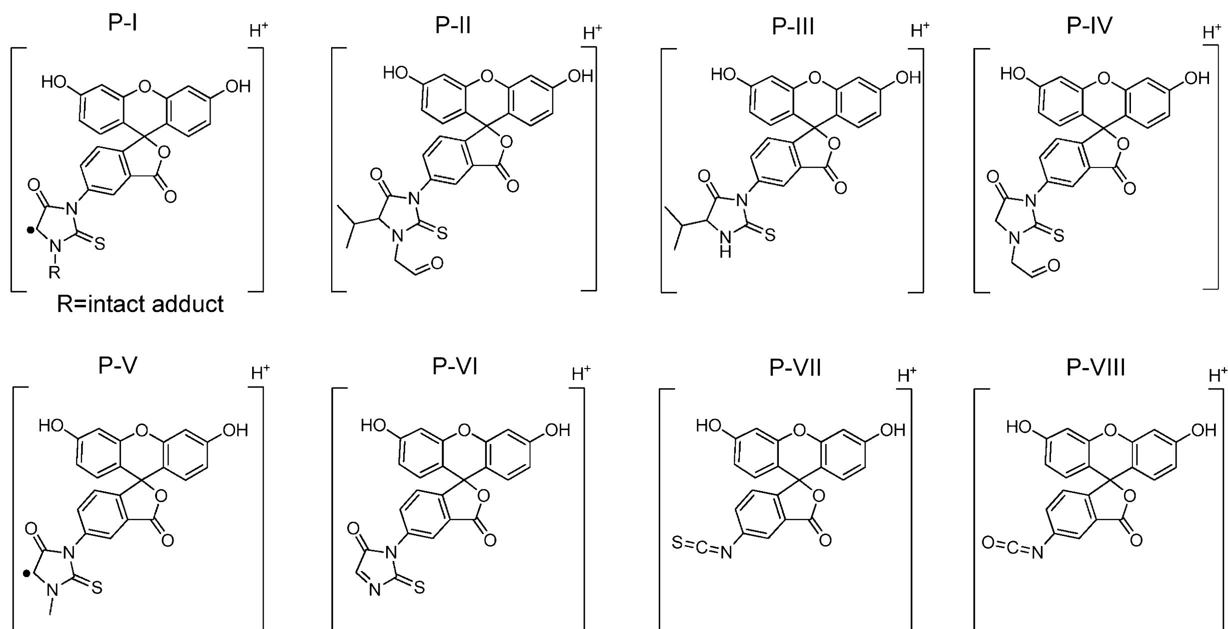

Fig. 2. Proposed product ions formed from the fragmentation data given in Table 2. Ions obtained by LC–MS/MS (ESI+) product ion scan.

solution containing FITC (90 mg, 0.23 mmol). The synthesis was performed as described for AA-Val-FTH but the purification of the product was done in three times smaller scale to yield 92 mg (0.16 mmol, 71%, pure according to TLC, Rf = 0.39, and LC–MS) as a solid compound. LC–MS/MS analysis, see Table 2 and Fig. 2.

2.4.11. Fluorescein-5-[4-isopropyl-3-(2-carbamoyl-2-hydroxyethyl)-2-thioxo-imidazolidin-5-one] (**4**, GA-Val-FTH)

The GA-Val-Na solution (1.0 mmol, see Section 2.4.4) was diluted with 0.125 M $KHCO_3$ (2 mL) reacted with FITC (195 mg, 0.50 mmol) dissolved in dioxan (1 mL). The synthesis and purification was done as described for d_3-AA-Val-FTH above. The yield after purification on silica gel column and drying in vacuo was 225 mg (0.39 mmol, 78%, pure according to TLC, Rf = 0.34 and LC–MS) as a solid compound. LC–MS/MS analysis, see Table 2 and Fig. 2.

2.4.12. Fluorescein-5-[4-isopropyl-3-(2-carbamoyl-2-hydroxy-1,1,2-d_3-ethyl)-2-thioxo-imidazolidin-5-one] (**5**, d_3-GA-Val-FTH)

The d_3-GA-Val-Na solution (0.20 mmol, Section 2.4.5) was diluted with 0.125 M $KHCO_3$ (1 mL) and reacted with FITC (58 mg, 0.15 mmol) dissolved in dioxan (0.5 mL). The synthesis and purification was done as described for AA-Val-FTH above. After purification on silica gel column the desired product was obtained to yield 62 mg (0.11 mmol, 72%, pure according to TLC, Rf = 0.34 and LC–MS) as a solid compound. LC–MS/MS analysis, see Table 2 and Fig. 2.

2.4.13. Fluorescein-5-[4-d_7-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one] (**6**, GA-d_7-Val-FTH)

The whole content of incubated GA-d_8-Val-Na solution (0.30 mmol, Section 2.4.6) was diluted with 0.125 M $KHCO_3$ (2 mL) and reacted with FITC (90 mg, 0.23 mmol) dissolved in dioxan (1 mL). The synthesis and purification was done as described for AA-d_7-Val-FTH above. The yield after drying in vacuo of the desired product was 105 mg (0.18 mmol, 78%, pure according to TLC, Rf = 0.34 and LC–MS) obtained as a solid. LC–MS/MS analysis, see Table 2 and Fig. 2.

2.4.14. Fluorescein-5-[4-isopropyl-3-(2-hydroxyethyl)-2-thioxo-imidazolidin-5-one] (**7**, EO-Val-FTH)

EO-Val (161 mg, 1.00 mmol) was dissolved in 0.5 M aqueous $KHCO_3$ (4 mL) and reacted with FITC (195 mg, 0.50 mmol) dissolved in dioxan (2 mL). The synthesis and purification was done as described for comp. **1**. The yield after purification on column and drying in vacuo was 223 mg (0.42 mmol, 84%, pure according to TLC, Rf = 0.55, and LC–MS) as a solid compound. LC–MS/MS analysis, see Table 2 and Fig. 2.

2.4.15. Fluorescein-5-[4-d_7-isopropyl-3-(2-hydroxyethyl)-2-thioxo-imidazolidin-5-one] (**8**, EO-d_7-Val-FTH)

The whole content of incubated EO-d_8-Val-Na solution (0.30 mmol, see Section 2.4.7) was diluted with 0.125 M $KHCO_3$ (1 mL) and reacted with FITC (90 mg, 0.23 mmol) dissolved in dioxan (0.5 mL). The synthesis and purification was done as described for comp. **3**. The yield after drying in vacuo was 84 mg (0.16 mmol, 68%, pure according to TLC, Rf = 0.55 and LC–MS) obtained as a solid compound. LC–MS/MS analysis, see Table 2 and Fig. 2.

2.5. Blood samples

The samples for analysis of Hb adducts were obtained from commercial human blood from Karolinska Hospital (Stockholm, Sweden). The blood was treated in three 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) before freezing. (c) Separated red blood cells were washed two times with 1.5 vol. of 0.9% NaCl solution before freezing. Defibrinated sheep blood from the National Veterinary Institute (Uppsala, Sweden) was used for calibration curves.

2.6. Equipment for sample preparation

The solid phase extraction (SPE) cartridges Oasis Max were obtained from Waters (Milford, Massachusetts, USA). A Ther-

momixer Comfort and a 5804 R centrifuge with rotor A-4-44 and F-45-30-11 (Eppendorf Nordic, Denmark) were used for preparation of analytical samples derivatised with FITC. The Hb analyzer (Hb 201+) was obtained from HemoCue (Ängelholm, Sweden).

2.7. Liquid chromatography–mass spectrometry

The LC–MS system consisted of a Shimadzu Prominence LC 20 system (Shimadzu Corp., Kyoto, Japan) interfaced to an API3200 Q-trap instrument with a TurboIonSpray® ionterface (ESI) obtained from Sciex (Concord, ON, Canada). A hypersil gold column was used (1.9 μm, 2.1 × 150 mm) (Thermo Fisher Scientific Inc., MA, USA) with a Trident guard filter (cap frits 0.5 μm 2 mm) (Restek., PA, USA). The mobile phase consisted of A: 0.1% formic acid in H_2O/ACN (4:1, v/v) and B: 0.1% formic acid in H_2O/ACN (1:4, v/v). A gradient was applied from 10% B to 70% B in 13 min, an isocratic flow in 5 min at 70% B and then stepped to 100% B in 5 min before re-equilibrating the column with the initial mobile phase. The injection volume was 20 μL and the flow rate was 100 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 gas (N_2) 5 au, ion spray voltage 4500 V and vaporizing temperature 400 °C. Analysis of processed samples was performed in the positive ion mode, using multiple reaction monitoring (MRM) with the following transitions: comp. **1** *m/z* 560.1 → 517.1; **2** *m/z* 567.1 → 517.1; comp. **3** *m/z* 576.1 → 531.1; **4** *m/z* 583.1 → 538.1; comp. **5** *m/z* 533.1 → 460.1; and comp. **6** *m/z* 540.1 → 460.1. The limit of quantification (LOQ) was set to 10 times the noise.

2.8. Calibration curve

Quantification was conducted using internal standard calibration with the hepta-deuterated FTH analytes (compound **3**, **6** and **8**). The calibration curve was established as the area ratios between analyte and internal standard versus added amount of analyte per sample. It was prepared by adding the standards, diluted in ACN:H_2O (3:7, v/v), as follows: 0.25, 0.50, 2.0, 4.0, 8.0 and 16.0 pmol/250 μL of sheep blood with Hb content of 130 g/L. The samples were then processed as described in Section 2.9. The added amount of internal standard was 5 pmol/250 μL of sample. The r^2 were consistently >0.99.

2.9. General procedure of the FIRE procedure

The Hb content in whole blood or isolated lyzed red blood cells was measured with a HemoCue instrument. Normally, whole blood (250 μL) was alkalized with 1 M $KHCO_3$ (15 μL), followed by addition of deuterium-substituted (d_7) FTH analytes. After addition of FITC (5 mg, 13 μmol) dissolved in DMF (30 μL), the sample was heated and mixed (37 °C at 800 rpm) on a thermomixer comfort for 16 h. Precipitation of proteins by adding ACN (1.6 mL) was performed under slow mixing, followed by centrifugation (10 min at 15,500 × *g*). A pH adjuster (25 μL, 1 M ammonium hydroxide) was added 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.

3. Results and discussion

3.1. Syntheses of reference compounds

The syntheses of the FTH analytes were done in a two-step reaction without isolation of the N-alkyl valines and where the final FTH analytes were purified. This procedure is fast and it gives rather high yields of the desired products. By using a slight excess of the electrophile (1.2 equiv.) and quite concentrated reaction mixtures in the first reaction, the main part of the valine is converted to the desired monoalkylated product and only to a low extent the dialkylated by-product (the depletion of valine was measured on TLC, the spots were developed with ninhydrin). In the following step, the derivatisation with FITC, between 0.5 and 0.75 mol equiv. of FITC were added to obtain complete reactions in the formation of FTH analytes of the N-alkylvalines. As FITC only reacts with primary and secondary amines (main yields) but not with tertiary amines, the desired products were obtained rather pure and could easily be separated by extraction. The product was purified from small amounts of reagent by-products, by column chromatography.

3.1.1. LC–MS/MS data of reference compounds

The adduct FIRE procedure was evaluated for the analysis of the adducts from acrylamide, glycidamide and ethylene oxide (AA-Val, GA-Val and EO-Val), at background levels in Hb. The LC–MS/MS conditions were evaluated using the synthesized FTH references of AA-Val, GA-Val and EO-Val (comp. **1**, **4** and **7**), which also were compared with obtained data from Val-FTH and MeVal-FTH, see [17]. The results showed that the structure of the adduct-substituents only has minor effects on the relative efficiency to ionize the FTH analytes (measured as small differences in LOQ). The obtained sensitivity for the reference compounds analyzed in SIM mode, expressed as LOQ, were 17 fmol ± 10% for compounds (**1**, **3**, **4**, **6–8**) in ESI positive ion mode used 0.1% formic acid in ACN/H_2O (1:1, v/v). This mobile phase condition facilitates the ionization and makes the analytes neutral which is beneficial for high retention on a C18 column. In the SIM mode the M + 1 ion was selected. In MRM mode the sensitivity differs mainly between the studied analytes due to the obtained intensity of the measured product ions (selected ion). The relative intensity and interpretation of formed fragments is presented in Table 2 and Fig. 2.

3.2. Development of the adduct FIRE procedure™

In the earlier studies pure standards of thiohydantoin derivatives of a few Edman reagents were compared regarding the relative sensitivity when measured by LC–MS/MS [16,17]. It was concluded that FITC was superior under all conditions tested which included different buffers and pH, mode of ionization and ionization techniques However, when utilizing this procedure on real samples with low adduct levels, a considerable development work had to be done in order to regain the sensitivity and to avoid pitfalls.

Studies were performed regarding the influence on the yields of the FTH analytes isolated from blood samples in relation to used amount of the FITC reagent and the time for derivatisation. To obtain optimal yields, it was obvious that the purity of the FITC reagent was crucial, therefore purification of this reagent as well as precautions during its storage were developed.

Considerable work was also performed for optimization of the overall procedure described in Sections 3.2.1–3.2.3 below. Different techniques for protein removal after incubation with FITC and different solid phase extraction procedures and HPLC columns have been studied (data not shown) and after revealing major pitfalls the selected procedure could be optimized. This work will be followed elsewhere by a complete validation of the described procedure.

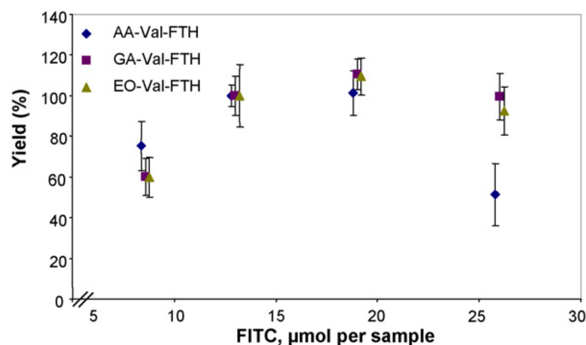

Fig. 3. Yields of FTHs of AA-Val, GA-Val, and EO-Val ($n = 3$) as a function of added amount FITC reagent.

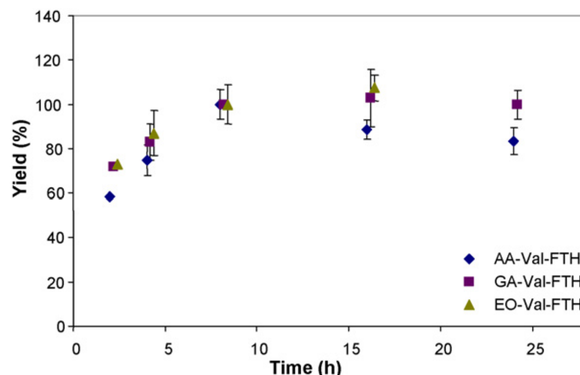

Fig. 4. Yields of FTHs of AA-Val, GA-Val, and EO-Val ($n = 3$) as a function of incubation time 2, 4, 8, 16 and 24 h.

3.2.1. FITC reagent

Dimetyl formamide (DMF) was found to be a good solvent for the reagent FITC allowing high concentrations. A solution of 13 µmol of FITC in 30 µL DMF was possible to add to 250 µL of lyzed whole blood without causing precipitation of the proteins. The amount of FITC used to achieve maximum yield varied to some extent for the three studied adducts. From the results shown in Fig. 3, it was concluded that 13 µmol FITC per 250 µL of blood was a suitable amount to be used in the procedure. It was observed that the two highest additions of FITC had a negative impact on the measured response of both the unsubstituted and deuterium-substituted analytes. The intensity decrease in the LC–MS/MS analysis was probably caused by ion suppression and not by degradation of the analytes. The samples with the highest additions of FITC were also difficult to resolve before LC–MS/MS analysis. The AA-Val-FTH analyte was more affected by high FITC additions than the GA-Val-FTH and EO-Val-FTH analytes, when the area ratios of the FTH analytes and internal standards were compared. The fact that FITC is soluble in whole blood at physiological pH is a necessary factor to make it possible to perform analysis of N-terminal adducts to Hb without pre-isolation of the globin.

3.2.2. pH modifier in the coupling reaction

The pH for the coupling reaction is a known critical factor for an effective thiohydantoin formation. The coupling reaction of isothiocyanate reagents to N-terminals is favoured by slightly alkaline conditions (pH about 8) while the ring formation is favoured by acidic conditions. Since whole blood was used in this study which has a buffer capacity itself, it was possible to perform the procedure without pH modifiers. Though, it was observed that an added amount of $KHCO_3$ equivalent to FITC increased the yield by 20%. This added amount is also consumed as FITC is added in its neutral spiro-form and it requires one equiv. base to be ionized and dissolved. More than 2 equiv. of $KHCO_3$ had a negative impact on the yields of FTH analytes.

3.2.3. Time and temperature for coupling and detachment

The time for derivatisation is also a factor that affects the yield of FTH analytes. In a time study 2, 4, 8, 16 and 24 h were tested. After 8 h (37 °C, 13 µmol FITC per 250 µL of lyzed whole blood, $n = 3$) a maximum yield was reached. A decrease in yield when proceeding for 16 or 24 h was only observed for the AA-Val-FTH analyte. The result for EO-Val-FTH at time 24 h was excluded due to problems with LC–MS/MS analysis (Fig. 4). Increasing the temperature speed up the reaction, but performing the reaction at 40 °C or higher was shown to cause clogging of the samples.

3.2.4. Clean-up procedure

To achieve an effective clean-up, different procedures were evaluated. Precipitation of proteins with an organic solvent (e.g. acetonitrile, ethanol or propanol) directly after the coupling/detachment reaction simplified further workup. The supernatant obtained after precipitation could be analyzed directly by LC–MS/MS (after evaporation and resolvation), with quantifiable peak intensities for all three analytes (**1**, **4** and **7**) in blood samples from non-smokers (data not shown). However, further clean-up was necessary as remaining FITC reagent and its by-products caused clogging of the analytical HPLC column and ion suppression. Therefore, one additional purification step was added. A mixed-mode anion exchange solid phase extraction column (SPE) (Waters MAX) gave the most efficient purification of the processed samples. A few other SPE columns and combinations were tested (data not shown).

When the samples were purified on SPE columns, it was shown that strong acids such as trifluoroacetic acid (TFA) should be avoided as the yields decreased in rather unpredictable ways. This decrease was found to be due hydrolysis of acid-sensitive adducts i.e., the amide group in AA-Val-FTH and GA-Val-FTH, which occurred during the preparation and storage of processed samples. Analytes without such groups, e.g. EO-Val-FTH, were found to be stable (data not shown). Tests with weaker acids than TFA (acetic acid, formic acid and cyanoacetic acid) showed that cyanoacetic acid ($pK_a = 2.5$) was strong enough to effectively release the FTH analytes from the ion exchangers without degrading acid-sensitive groups.

Another pitfall for the success of the procedure was the use of methanol in the SPE clean-up procedure (which is a recommended solvent by the SPE supplier). Methanol in combination with used acids, especially TFA, had a general negative effect on peak heights in the MS (data not shown). This side-reaction was not further studied but a probable explanation is that alcohols and acids lead to esterification of the carboxyl group in the fluorescein moiety. By instead using ACN in the SPE clean-up procedure, this side-reaction was avoided.

3.2.5. HPLC separation

During the course of the development a number of different analytical reversed phase (C_{18} and C_8) HPLC columns were tested (data not shown). With the used equipment the obtained sensitivity was gained by using relatively long columns and small particle sizes (100–150 mm and particle sizes down to 1.9 µm), which had inner diameters of either 2.1 or 1.0 mm. It was found that columns with high carbon load, providing stronger retention of the FTH analytes, gave the highest sensitivity in the MS/MS analysis (observed as higher peaks and s/n-ratios). Reasons for the gain in sensitivity could not alone be explained by a better peak-shape (concentration dependence), but rather due to a more efficient ionization of the FTH analytes as they eluted at higher ACN concentration when

Table 3
Hb adduct levels of glycidamide, acrylamide and ethylene oxide in human blood samples processed from; whole blood, blood erythrocytes or washed erythrocytes by the FIRE procedure. All samples were lyzed by freezing before processed.

Hb adducts	Blood donor	Matrix		
		Whole blood ($n=3$) pmol/g Hb (SD)	Erythrocytes ($n=3$) pmol/g Hb (SD)	Washed erythrocytes ($n=3$) pmol/g Hb (SD)
Glycidamide	1	18.4 (1.5)	20.3 (1.3)	21.0 (1.2)
	2	19.9 (0.7)	20.3 (2.5)	22.0 (1.0)
Acrylamide	1	47.7 (1.8)	44.5 (0.8)	46.5 (2.1)
	2	34.5 (1.4)	38.9 (0.5)	34.4 (2.5)
Ethylene oxide	1	16.5 (1.7)	15.5 (2.6)	17.9 (1.5)
	2	19.1 (1.6)	15.6 (2.8)	15.1 (2.0)

using high carbon load columns. The usage of a pre-column filter is highly recommended as small particles from the sample otherwise will cause increased column pressure resulting in reduced life span of the columns.

3.3. Stability of formed FTH analytes

A stability test was performed for stored processed samples, solved in dioxane:H_2O (3:7, v/v). An average decrease of 40% ($n=3$) was obtained for the GA-Val-FTH and AA-Val-FTH analytes when the samples were stored at 37 °C for 7 days. No significant decrease was observed for the EO-Val-FTH analyte under the same conditions. No significant difference was obtained for samples stored 7 days at room temperature, or in a refrigerator at 6 °C ($n=3$). No shift of the ratio between the analyte and the internal standard was observed for the GA-Val-FTH, AA-Val-FTH or EO-Val-FTH analytes during storage in refrigerator, room temperature or at 37 °C.

3.4. Analysis of human blood samples

An example of the LC–MS/MS (MRM, ESI, positive ion mode) results from a human blood sample is presented in Fig. 5. The limit of quantification (LOQ) for processed samples when starting from 250 μL of blood with a concentration of Hb of 130 g/L were: 1 pmol/g Hb for GA-Val-FTH, 2 pmol/g Hb for AA-Val-FTH and 2 pmol/g Hb for EO-Val-FTH. The results on the quantified Hb-adduct levels in two individuals are shown in Table 3 where the derivatisation was done either by adding FITC directly in whole blood, to isolated erythrocytes or to isolated and washed erythrocytes, respectively. These samples were then processed by the FIRE procedure for measurement of three analytes; AA-Val-FTH, GA-Val-FTH and EO-Val-FTH. The results indicate that isolation and/or purification of erythrocytes before derivatisation only has a minor effect, if any, on the measured adduct levels. This shows that it is possible to analyse these adducts direct in whole blood which can simplify sample processing but also opens up for new applications to biobanks where only whole blood is stored. The measured adduct levels is in accordance with previously reported data for non-smokers [e.g. refs. 8–15,18,19].

When performing the optimized procedure (see Section 2.9) the total recovery for the FTH standards added to un-processed human blood (5 pmol/250 μL sample, $n=6$) was 62% (5.5% RSD) for GA-Val-FTH, 72% (6.5% RSD) for AA-Val-FTH and 67% (6.7% RSD) for EO-Val-FTH. The obtained recoveries were adjusted for the background adduct levels. The major losses were obtained in the SPE clean-up step, these contributed to the total loss with; 62% (10% RSD) for GA-Val-FTH, 63% (20% RSD) for AA-Val-FTH and 61% (17% RSD) for EO-Val-FTH. Only minor losses occurred in the protein precipitation step and during the concentration step prior analysis. By using ion-exchange SPE a less adduct discriminating clean-up procedure was achieved, since the properties of the adduct moiety would be of little importance. Despite the

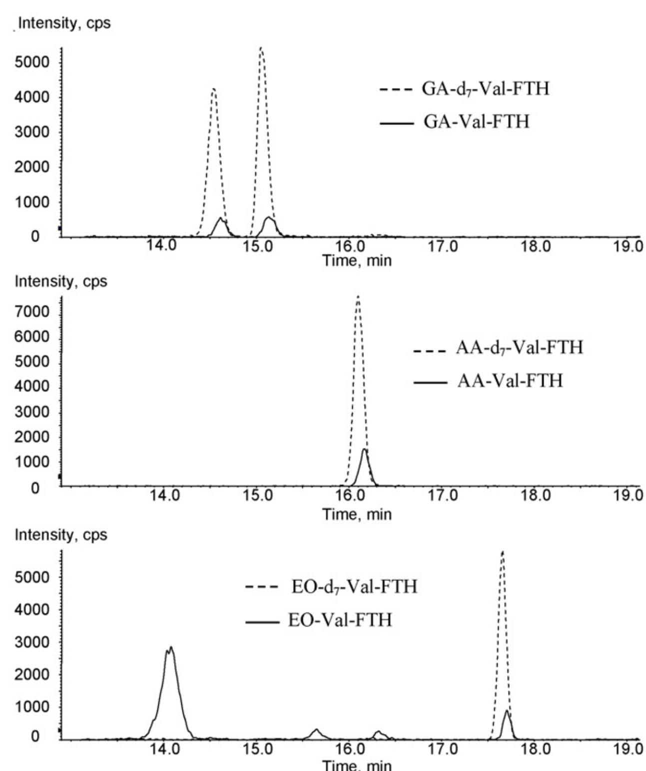

Fig. 5. LC–MS/MS chromatograms with MRM transitions for GA-Val, AA-Val and EO-Val (FTHs) versus IS from a blood sample of a non-smoker.

clean-up of samples, a matrix effect in the analysis by LC–MS/MS could not totally be avoided. For studying matrix effect processed samples were spiked before LC–MS/MS analysis and the response (area) was compared with an analytical standard solution (1 pmol injected/analyte, $n=6$). The matrix effect was estimated to be; 18% (2.2% RSD) for GA-Val-FTH, 8% (1.7% RSD) for AA-Val-FTH and 15% (3.7% RSD) for EO-Val-FTH.

4. Conclusions

A state of the art method is described for measurement of N-terminal Hb adducts of acrylamide, glycidamide and ethylene oxide at background exposure levels in human blood. The new method using FITC as the Edman reagent opens up for derivatisation direct in whole blood and as the reagent is ionized at neutral pH the clean-up procedure of formed thiohydantoins could be simplified significantly by using protein precipitation, and purification of the analytes on a mix mode SPE column with ion exchanger. The adduct FIRE procedure^{TM} was shown to be specific and stable and it has the potential to be a valuable tool for measurement of a broad range of Hb adducts occurring at low levels. The sensitivity of the

method was shown to be high and rather constant (LOQ, were 17 fmol ± 10%) for the studied analytes (six compounds compared). The short analytical chain including purification on SPE columns has increased the sample throughput considerably. The used procedure is also suitable for automatization to be used for large multi analyte screening studies. Samples stored in biobanks as whole blood could be used for adduct determination, and samples collected for future studies do not need to be specifically processed (isolation and washing of erythrocytes) for adduct determination, before storage.

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