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Identification of covalent binding sites of ethyl 2-cyanoacrylate, methyl methacrylate and 2-hydroxyethyl methacrylate in human hemoglobin using LC/MS/MS techniques☆

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ABSTRACT

Acrylates are used in vast quantities, for instance in paints, adhesive glues, molding. They are potent contact allergens and known to cause respiratory hypersensitivity and asthma. Here we study ethyl 2-cyanoacrylate (ECA), methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA). There are only limited possibilities to measure the exposure to acrylates, especially for biological monitoring. The aim of the present study was to investigate the chemical structures of adducts formed after reaction of hemoglobin (Hb) with ECA, MMA, and HEMA. This information may be used to identify adducted Hb peptides for biological monitoring of exposure to acrylates. Hb-conjugates with ECA, MMA, and HEMA were synthesized *in vitro*. The conjugates were digested by trypsin and pronase E. Adducted peptides were characterized and analyzed by liquid chromatography and nano electro spray/hybrid quadrupole time-of-flight mass spectrometry (MS) as well as tandem quadrupole MS. The search for the adducted peptides was facilitated by visualizing the MS data by different computer programs. The results showed that ECA binds covalently to cysteines at the 104 position in the α and the position 112 in the β-chains in Hb. MMA and HEMA bound to all the cysteines in both chains, Cys^{104} in the α-chain and Cys^{93} and 112 in the β-chain. The full-length spectra of in un-digested Hb confirmed this binding pattern. There was no reaction with N-acetyl-L-lysine at physiological pH. The adducted peptides were possible to measure using LC/MS/MS in selected reaction monitoring mode. These peptides may be used for biological monitoring of exposure to ECA, MMA and HEMA.

1. Introduction

Acrylates are derivatives of acrylic acid (Fig. 1). The acrylates polymerize readily at the vinyl group, catalyzed by, e.g., light, heat, and oxygen. The acrylates are industrially important and used in vast quantities in for instance in paints, adhesive glues, molding. In this study we investigate two groups of acrylates, cyanoacrylate and methacrylate. Ethyl 2-cyanoacrylate (ECA) is highly reactive and polymerizes rapidly, while methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA) are less reactive.

Numerous articles have been published concerning the sensitizing potential of the acrylates. ECA, MMA and HEMA are potent contact allergens [1–3]. They are also known to cause respiratory hypersensitivity and asthma [4–7].

Occupational exposures to acrylates are common among large numbers of workers in car, printing, plastic, and electronic industry, but also among health care and cosmetic personnel. However, there is very little information about the exposure to acrylates since only methods for measuring a few acrylates are available. Some measurements of acrylates in air and on dermal patches have been described [8–10].

Biological monitoring has several advantages compared to environmental sampling; e.g., it measures the internal exposure which compensates for dermal uptake and different work-load. Previously, only a few studies have been performed on biomarkers of exposure to acrylates. Mizunuima et al. [11] analyzed methanol as a biomarker for MMA exposure, but methanol is not a specific biomarker. Studies in rats have shown that methacrylates are hydrolyzed by carboxylesterase forming methacrylic acid after exposure [12]. Furthermore, it has also been shown in a rat study that MMA binds covalently to glutathione by a Michael addition reaction and is excreted as a mercapturic acid

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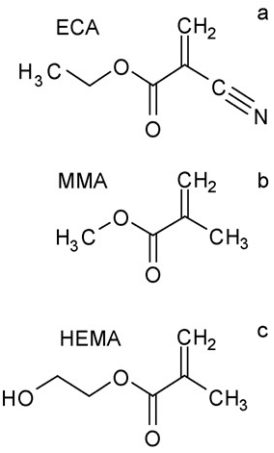

Fig. 1. Chemical structure of (a) ethyl 2-cyanoacrylate (ECA), (b) methyl methacrylate (MMA), and (c) 2-hydroxyethyl methacrylate (HEMA).

in urine [13]. However, neither methacrylic acid nor the mercapturic acids have been measured in human studies. Thus, there is a need for deeper knowledge of the biotransformation of the acrylates in humans after exposure and for development of new biomarkers.

In a rat study it has been shown that acrylates can form protein adducts [14]. *In vitro* studies have shown that lysine and cysteine reacts with acrylates [15]. Protein adducts have been used as an index of long-term exposure which was first suggested by Ehrenberg et al. [16]. It has previously been shown that Hb adducts can be used as long-term biomarkers of sensitizing compounds [17,18]. Protein adducts formed by sensitizing chemicals have been characterized in several studies [17,19–23] using LC/MS/MS or GC/MS. However, the identity of covalent binding sites of acrylates in proteins has not previously been studied. This knowledge can be used to find peptides useful for biological monitoring.

The aim of the study was to investigate the chemical structures of adducts formed after *in vitro* reactions of Hb with ECA, MMA, and HEMA. This information may be used to identify adducted Hb peptides, after enzymatic digestion of the conjugates, that can be applied as biomarkers of exposure to acrylates.

2. Experimentals

2.1. Materials

NaCl, KCl, KH_2PO_4, Na_2SO_4, $CaCl_2$, NaOH, formic acid, acetone, HCl and CH_3COONH_4 were purchased from Merck (Darmstadt, Germany). $Na_2HPO_4{\cdot}12H_2O$, Trifluoroacetic acid (TFA), pronase E, CsI, N-acetyl-L-lysine, methyl methacrylate, 2-hydroxyethyl methacrylate and ethyl 2-cyanoacrylate were purchased from Sigma–Aldrich Chemicals (Steinheim, Germany). Acetonitrile and methanol were purchased from Lab-scan (Dublin, Ireland). $Na_3PO_4{\cdot}12H_2O$ was purchased from Jansen Chimica (Geel, Belgium). Triethylamine was purchased from Acros Organics (Geel, Belgium). Dithiothreitol (DTT) and iodacetamide were purchased from ICN Biomedicals (Aurora, OH, USA) and were used for disulphide reduction and carboxyamidomethylation, respectively. Dialysis membrane MW cut off 3500 Da was purchased from Spectrum (Gardena, CA, USA). Trypsin (sequencing grade) was purchased from Roche Diagnostics Gmbh (Mannheim, Germany). Zip tip C_{18} pipettes were purchased from Millipore (Bedford, MA, USA). Sex pheromone inhibitor iPDI, H9985 for calibrating the hybrid quadrupole time-of-flight MS (QqTOF), was purchased Bachem (Bubendorf, Switzerland). Water was purified to an ultra-pure grade by an ELGA Maxima HPLC Mark II (USFELGA, Bucks, UK) purification system.

2.2. Instrumentation

Qualitative analyses of enzymatic digests of the conjugates were performed using a reversed phase liquid chromatography (LC) system equipped with a capillary and nano pump (nanoLC; 1100 series; Agilent Technologies, Santa Clara, CA, USA). The sample was trapped on a C_{18} trap column (0.3 mm × 5 mm, 5 μm, Zorbax 300SB C18, Agilent Technologies) using the capillary pump and then transferred and analyzed on a C_{18} column (0.1 mm × 150 mm, 3.5 μm, Zorbax 300SB C18, Agilent Technologies) using the nano pump. The LC system was coupled online to a fused-silica PicoTip (50 μm i.d. × 360 μm o.d. × 8 μm tip, New Objective, Woburn, MA, USA) using a nanoelectrospray source (Proxeon, Odense, Denmark) connected to a nano electro spray/hybrid quadrupole time-of-flight mass spectrometry (QqTOF MS; QSTAR pulsar; Applied Biosystems, Foster City, CA, USA).

Characterization of un-digested Hb and Hb-conjugates (full-length protein) and selected peptides were performed using offline nanoelectrospray tips (nanoES; Proxeon Biosystems, Odense, Denmark and New Objective, Woburn, MA, USA) connected to the QqTOF MS (nanoES-QqTOF; Applied Biosystems). The QqTOF was calibrated daily using CsI (m/z 132.9054) and the pentapeptide iPDI (m/z 829.5398).

Analysis of adducted peptides and N-acetyl-L-lysine acrylate adducts was performed using triple quadrupole MS equipped with a turbo ionspray source (API 3000, Applied Biosystems) coupled to a LC system (LC/MS/MS; Perkin Elmer, Norwalk, Connecticut, USA). The samples were injected on a C_{18} column (4 μm, 2.1 mm i.d. × 50 mm GENISIS; Grace Vydac, Hesperia, CA, USA) and analyzed in the positive ion mode. The samples were analyzed in MS scan mode or single reaction mode (SRM). The temperature in the ion source was set at 350 °C. Pure nitrogen was used as nebulizer, auxiliary, curtain and collision gas.

2.3. Test of reactivity between N-acetyl-L-lysine and ECA, MMA or HEMA

Solutions containing 10 nmol N-acetyl-L-lysine were prepared in 1 ml of PBS (pH 7.4). The acrylates were dissolved in dry acetonitrile and added in drops to the samples. ECA was added in a molar ratio of 1:5 (amino acid:ECA). MMA and HEMA were added in the molar ratio of 1:100. As a catalyst, triethylamine was added to the MMA and HEMA mixtures in the molar ratio 1:10. The samples were incubated for 24 h at 37 °C before analysis.

Analysis of the samples was performed using LC/MS/MS. Five microliters of each sample was injected on a C_{18} column. The flow rates of the mobile phases were 0.3 ml/min of water (A) and 5% methanol (B) both acidified with 0.5% acetic acid. The samples were separated using a gradient elution to 95% B during 5 min. The LC/MS/MS was operated in single MS scan mode.

2.4. In vitro synthesis of conjugates between hemoglobin and ECA, MMA, or HEMA

A scheme of the complete procedure in the work is shown in Fig. 2. Blood obtained from a donor was used to prepare Hb from erythrocytes. The erythrocytes were washed three times with 0.9 mg/ml NaCl, lyzed with water and stored at −20 °C. Four aliquots of Hb solution (300 mg) diluted in 1 ml of PBS (pH 7.4) were prepared, one for each conjugate and one Hb-blank sample. The acrylates were dissolved in dry acetonitrile and added in drops to the Hb-samples. The Hb-blank sample was handled equally but without addition of the acrylates. The conjugates were made

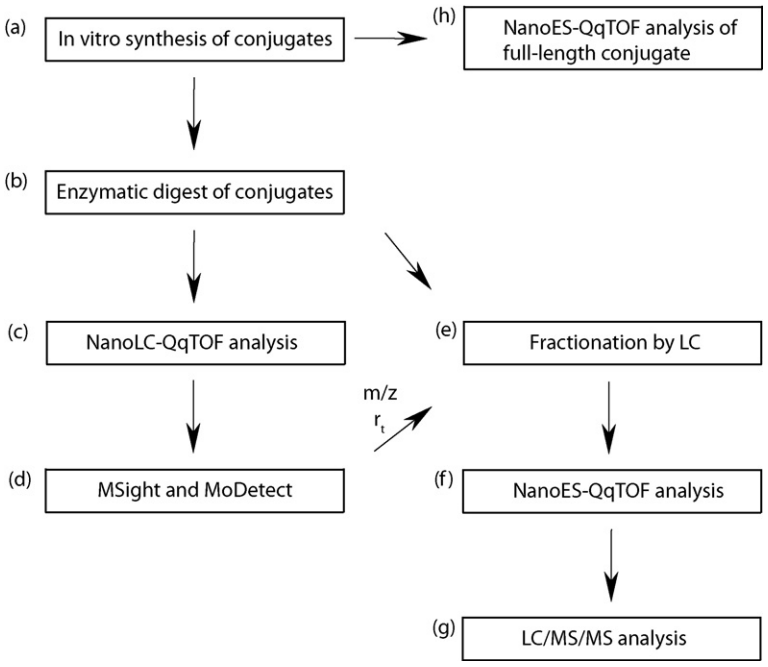

Fig. 2. Scheme of the procedures used in the study. (a) *In vitro* conjugates between hemoglobin (Hb) and the different acrylates were synthesized. (b) These were digested by either trypsin or pronase E. (c) The enzyme digests and a Hb-blank were then separated and analyzed by nano liquid chromatography coupled to a nano electro spray/hybrid quadrupole time-of-flight mass spectrometry (nanoLC-QqTOF). (d) The data from the nanoLC-QqTOF were then subjected to data analyses using the computer program MSight and MoDetect. (e) The enzyme digested conjugates were fractionated using liquid chromatography (LC) methods. (f) Selected fractions from these LC separations were subjected to analyses by offline nano electro spray/hybrid quadrupole time-of-flight mass spectrometry (nanoES-QqTOF). The mass to charge ratios (m/z) and retention times (r_t) obtained by MSight and MoDetect were used to select fractions for further analyses. (g) Adducted peptides, identified in the nanoES-QqTOF analyses, were then used to develop method for LC triple quadrupole mass spectrometry in the selected reaction monitoring mode (LC/MS/MS). (h) In addition, the full-length conjugates are analyzed by nanoES-QqTOF which identify the total number of adducts attached to the α and β Hb-chains.

at the molar ratios: Hb:ECA, 1:5, Hb:MMA, 1:50 and Hb:HEMA, 1:100. To facilitate the reaction between Hb and MMA or HEMA, triethylamine in a molar ratio of 1:10 was added to the reaction mixture. The acrylate incubated Hb-samples as well as the blank Hb were incubated for 24 h at 37 °C and thereafter dialyzed for 2 days against PBS and then 5 days against 50 mM CH_3COONH_4 as previously described for *in vitro* synthesis of conjugates between hb and hexahydrophthalic anhydride [21]. All samples were stored at −20 °C until further use.

2.5. Work up procedures

Dialysed protein solutions of the three conjugates and the Hb-blank were precipitated using cold acetone containing 0.1 M HCl, and thereafter washed three times with cold acetone. The acetone was evaporated and the dry protein was stored at −20 °C. Prior to use, Hb–ECA, Hb–MMA, and Hb–HEMA conjugate and Hb-blank were dissolved in 1.0 ml water (2.5 mg/ml). The samples were reduced with DTT (3.1 mg) for 1 h at 55 °C and then carboxyamidomethylated for 30 min in darkness and at room temperature using iodacetamide (6.3 mg). This was performed to avoid oxidation of the free cysteine residues that otherwise can spontaneously form new disulphide bonds. These di-peptides caused problems in the computer analysis of nanoLC-QqTOF data (see below) when comparing conjugates and Hb-blank samples.

Trypsin dissolved in 0.05 mM HCl, 25 mM CH_3COONH_4 and 1 mM $CaCl_2$ was added in 1:50 weight ratio (trypsin:conjugate/blank). The samples were incubated for 16 h at 37 °C, thereafter evaporated to dryness and stored at −20 °C.

Pronase E dissolved in 50 mM CH_3COONH_4 was added in a 1:10 weight ratio (pronase E:conjugate/blank). The samples were incubated for 4 days at 37 °C and each day a fresh solution of pronase E in 1:10 ratio was added. The samples were evaporated to dryness and stored at −20 °C.

2.6. NanoLC-QqTOF analysis of trypsin digested Hb–ECA, Hb–MMA, and Hb–HEMA conjugate

The analysis of the trypsin digested conjugates was performed using nanoLC-QqTOF. Four microliters (0.08 μg) of sample was loaded onto the trap column using the capillary pump with the flow rate of 10 μl/min water containing 3% acetonitrile and 0.1% formic acid. The valve shifted after 6 min and the sample eluted onto the C_{18} analytical column using a 90 min gradient on the nano pump at a flow rate of 300 nl/min. The mobile phases used were water (A) and acetonitrile (B) both acidified with 0.1% formic acid. The gradient started at 5% B and continued to 70% B where the mobile phase was kept isocratic for 10 min before returning to 5% B. The QqTOF was set to collect TOF spectra for 130 min between m/z 200 and 1400. The Hb-blank was run before the Hb-conjugate. In addition, to minimize the risk of carry over effect, water was run between each sample.

2.7. NanoLC-QqTOF analysis of pronase E digested Hb–ECA, Hb–MMA, and Hb–HEMA conjugate

The analysis of the pronase E digested conjugates was performed using nanoLC-QqTOF. The trap column was replaced with a loop with the volume of 2.9 μl. A total of 6 μl of sample (0.15 μg) was injected to fill the loop using the capillary pump with the flow rate of 10 μl/min of water with 3% acetonitrile and 0.1% formic acid. The valve shifted after 1.2 min and the sample eluted onto the C_{18} analytical column using a 40 min gradient on the nano pump at a flow rate of 300 nl/min. The mobile phases used were water (A) and acetonitrile (B) both acidified with 0.1% formic acid. The gradient

started at 2% B and continued to 90% B where the mobile phase was kept isocratic for 5 min before returning to 2% B. TOF spectra were collected for 70 min between m/z 70 and 750. The Hb-blank was run before the Hb-conjugate. In addition, to minimize the risk of carry over effect, water was run between each sample.

2.8. Computer analysis of nanoLC-QqTOF data of the trypsin digested conjugates

To identify adducted peptides in the nanoLC-QqTOF data, two different softwares were used, MSight, freeware available at http://www.expasy.org/MSight and MoDetect, developed in house at our department and used for the first time in the present work.

MSight 2.0 was developed by the Proteome Informatics Group and has been described by Palagi et al. [24]. Prior to the data analysis the nanoLC-QqTOF data files were converted into mzWiff file format. MSight was then used to visualize the nanoLC-QqTOF data as 2D images. The vertical dimension (y-axis) represents the retention time obtained from the LC separation, while the horizontal dimension (x-axis) represents the m/z values from the MS. The intensity of the images corresponds to the MS signal intensities. To evaluate differences in the nanoLC-QqTOF data, each conjugate was compared to a Hb-blank sample. These images were then warped and aligned with the use of landmarks to compensate differences in elution times. The 2D images were then displayed in a transparency mode on top of each other to facilitate the comparison of the two data sets. The m/z and charge of the peptides that were visualized in the conjugates data files but not in the Hb-blank data files were further analyzed using nanoES-QqTOF MS (described below).

The MoDetect software requires the nanoLC-QqTOF data files to be converted into TAB-delimited files before analysis. MoDetect accommodated data into a three column grid including, retention time, m/z, and signal intensity and then groups all retention times into intervals of 1 min. A one-dimensional vector is allocated into the memory and the m/z values are placed in the vector at the element corresponding to the retention time. To compensate for differences in the retention time the grouped 1 min data were also compared with the adjacent grouped data. The software could adjust for differences in retention times of ±3 min. The chemical and instrumental noise level was compensated by a signal cut off at 50 cps. To evaluate differences in the nanoLC-QqTOF data, each conjugate was compared to a Hb-blank sample. MoDetect lists the m/z from the conjugate that differentiate from the blank sample and these were further analyzed using nanoES-QqTOF MS (described below).

2.9. NanoES-QqTOF analysis of tryptic digests

To minimize ion suppression effects in the nanoES-QqTOF analysis, trypsin digested conjugates were separated and fractionated by LC prior to analysis using a C_{18} column. One mg of tryptic digests of each conjugate was dissolved in 50 μl of water. Two minutes fractions were collected in a total of 45 fractions, as described earlier [21]. The fractions were evaporated to dryness and dissolved in 20 μl of water. An aliquot of 7 μl was acidified with 2.5 μl of 2.5% TFA, and further purified using C_{18}-zip tips [23] prior to analysis using nanoES-QqTOF. The TOF spectra were obtained between m/z 100 and 2000 and product ion scan spectra were obtained between m/z 100 and 1600. The sequence from Hb was obtained from SwissProt P66905 (α-chain) and P68871 (β-chain). Hb was cleaved *in silico* using tools in Analyst QS 1.1.

2.10. NanoES-QqTOF analysis of pronase E digests

Pronase E digests of conjugates and Hb-blank were mixed with a solvent mixture containing 30% formic acid, 40% methanol and 30% water analyzed using the nanoES-QqTOF. MS and product ion scan spectra were collected between m/z 70 and 750.

2.11. NanoES-QqTOF analysis of full-length Hb–ECA, Hb–MMA, and Hb–HEMA conjugate

Analysis of full-length Hb–ECA, Hb–MMA, and Hb–HEMA conjugates were performed using nanoES-QqTOF. The precipitated conjugates were dissolved in water and a few microliters was mixed with a solvent mixture containing 30% formic acid, 40% methanol and 30% water. The TOF mass spectra were collected between m/z 600 and 1600. The software Biospec Reconstruct from Applied Biosystems was used to transform the mass spectra of multiply charged ions into the molecular mass of the macromolecules.

2.12. Analysis of trypsin and pronase E digested adducted conjugates by targeted SRM LC/MS/MS

Ions corresponding to acrylate adducted peptides were obtained from the nanoES-QqTOF data of trypsin and pronase E digested Hb–ECA, Hb–MMA, and Hb–HEMA conjugates. These were used to obtain suitable SRM transitions for LC/MS/MS analysis. The sample (15 μl) was injected on a C_{18} column. Water (A) and methanol (B), both acidified with 0.5% acetic acid, was used as mobile phase. The samples were separated using a gradient with 5–99% B during 10 min for the trypsin digested peptides and 5 min for the pronase E digest and the column was then re-conditioned at 5% B for 2 min. The flow rate was set at 0.3 ml/min. A Q1 scan was performed to select $[M+2H]^{2+}$–$[M+5H]^{5+}$ ions corresponding to the adducted peptides. In a second step a product ion scan was performed for each ion in order to select the fragments with the highest abundances. The collision energies (CE) and declustering potentials (DP) were optimized in the SRM method by infusion of the digests of the conjugates. Standards were obtained from weighted amounts of the conjugates.

3. Results and discussion

3.1. Synthesis of conjugates

ECA reacted rapidly and formed adducts at low molar ratios, whereas MMA and HEMA reacted slowly and high molar ratios and triethylamine as a catalyst were required before adducts were formed. Other studies have shown that acrylates react with nucleophilic amino acids in a Michael addition reaction [15,25]. In an animal study it has been shown that acrylates can form protein adducts [14]. However, covalent binding sites of acrylates on Hb or other proteins have not been studied previously.

3.2. Computer analysis of trypsin digestets of nanoLC-QqTOF data

The visualized 2D image for the trypsin digested Hb–ECA conjugate indicated a rather complex adduct formation. The trypsin digested Hb–MMA and Hb–HEMA conjugate indicated a less complex pattern with only a few modifications more than the Hb-blank. In Fig. 3, 2D images of trypsin digested Hb–HEMA and Hb-blank after alignment and visualization using MSight are shown. The MoDetect software produced a list of all ions that differed between the trypsin digested conjugates and Hb-blank. MoDetect identified all ions found using MSight as well as a few new peptides. All peptides from the acrylate conjugates that differed from the Hb-blank were selected for further analysis using nanoES-QqTOF. For these ions, information on m/z, charge and approximate retention time were used in the further analysis (see Section 3.3).

The nanoLC-QqTOF analysis produced very large data sets that were processed and analyzed using the softwares MSight and MoD-

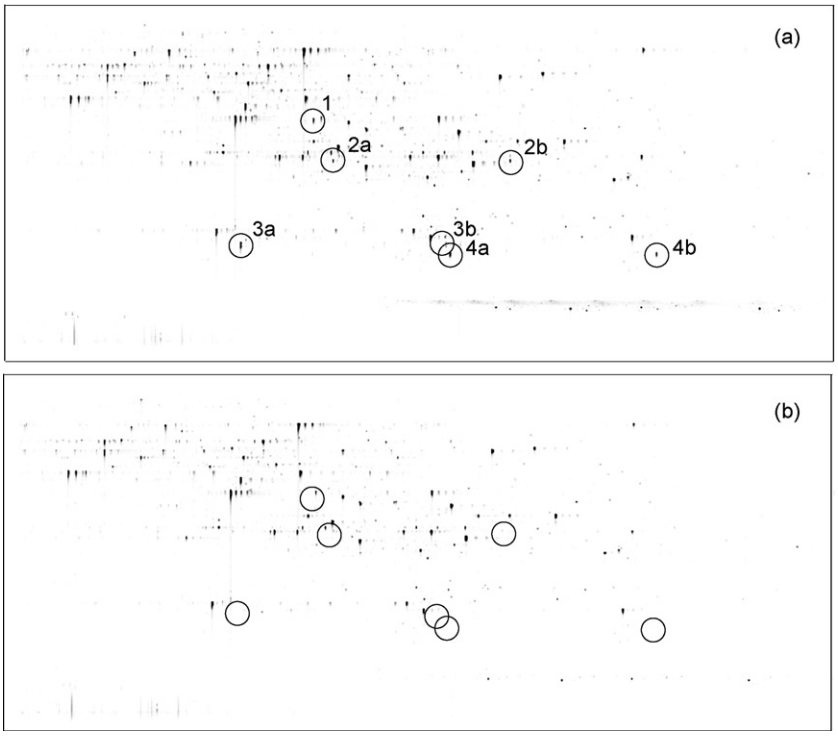

Fig. 3. Visualized nanoLC-QqTOF data using MSight. (a) 2D image of the Hb–HEMA conjugate. The spots corresponding to the tryptic HEMA adducted peptides have been indicated as follows; 1: β 83–95 $[M+130+3H]^{3+}$, m/z 517.95; 2a: β 83–104 $[M+130+5H]^{5+}$, m/z 532.60; 2b: β 83–104 $[M+130+4H]^{4+}$, m/z 665.60; 3a: β 105–120 $[M+130+4H]^{4+}$, m/z 463.20, 3b: β 105–120 $[M+130+3H]^{3+}$, m/z 617.40; 4a: α 100–127 $[M+130+5H]^{5+}$, m/z 620.35; 4b: α 100–127 $[M+130+4H]^{4+}$, m/z 775.21. (b) 2D image of Hb-blank.

etect to target adducted peptides. Visualizing the nanoLC-QqTOF data as 2D images using MSight facilitated the search for the adducted peptides by comparing trypsin digested conjugates and Hb-blank samples. In previous work we have used more time consuming strategies such as acid hydrolysis of the different fractions followed by analysis of the released adducts or manual comparison [21,23,26].

3.3. NanoES-QqTOF analysis of trypsin digested conjugates

The tryptic digest of Hb–ECA, Hb–MMA, and Hb–HEMA conjugate were separated and fractionated into 45 fractions. The approximate retention times, obtained from the nanoLC-QqTOF analysis, were used to select the fractions for nanoES-QqTOF analysis. In the selected fraction, TOF spectra were collected and ions with the selected m/z from MSight and MoDetect were subjected to MS/MS to identify the peptide. Fractions 10–44 were analyzed for the Hb–ECA conjugate and fractions 24–39 were analyzed for the Hb–MMA conjugate and the Hb–HEMA conjugate. Peptides observed with an additional mass or a multiple of 125 or 97 Da corresponded to ECA adducts. The 97 adduct comes from ECA which has lost the ethyl group due to carboxylesterase activity. It has previously been described that carboxylesterase can hydrolyze the ester of the acrylates into acrylic acids [12,13]. Furthermore, the peptides with an additional mass of 100 Da corresponded to MMA adducts and 130 Da to HEMA adducts. The b- and/or y″-series ions and immonium ions were the most prominent fragment series ions observed, and these pinpointed adducts between the amino acid and acrylates. The determination of the sequence and adducted amino acid was done as described by Jeppsson et al. [23]. Several of the ions in the trypsin digested conjugates differing from the Hb-blank samples did not correspond to adducted peptides. These were mainly peptides also found in the control but with a much lower intensity suggesting discrepancy in ionization response for the trypsin digested conjugate sample or in the efficiency of the trypsin digestion. Others were identified as tryptic misclevages in the conjugate samples and were not found or only found in low extent in the Hb-blank sample. These were seen in all acrylate conjugates but were more abundant in the Hb–ECA conjugate. One of the adducted peptides is described by the b-ions, y″-ions, and the immonium ions. The adducted amino acid will be denoted with an *. All other adducted peptides are described in supplementary data.

Peptide β 105–120 (LLGNVLVC*VLAHHFGK; $[M+130+3H]^{3+}$, m/z 617.40). The peptide fragmented into a singly charged y"-series ions with peaks from $y''_1 - y''_8$. From $y''^{*}_9 - y''^{*}_{11}$ all ions were modified by the mass of 130 Da. The fragment ion y''^{*}_9 (m/z 1141.60) identified Cys^{112} as the HEMA adducted amino acid. The N-terminal was identified with b-series ions b_2–b_5. The immonium-ion of cysteine–HEMA (m/z 206.11) was identified. The spectrum is shown in Fig. 4. The results for the other adducted tryptic peptides are shown in Table 1.

3.4. NanoES-QqTOF of pronase E digested conjugates

Data from the nanoLC-QqTOF analysis of pronase E digested conjugates were analyzed using the softwares MSight and MoDetect to identify ions that potentially were adducted amino acids or peptides. Both softwares identified the same ions and these were further analyzed using the nanoES-QqTOF. The ion found in the Hb–ECA conjugate was m/z 443.07, corresponding to ValCys with an additional mass of 222 Da ($ECA^{97} + ECA^{125}$). The dipeptid ValCys corresponds to Cys^{112} and either Val^{111} or Val^{113} within the β-chain. The product ion spectra showed a peak for the ValCys-adduct and a peak for adducted cysteine (m/z 344.0) losing the valine during fragmentation. This clearly shows that it is the cysteine that is adducted. In addition, it is also clear from these results that ECA has been polymerized and there are two ECA molecules bound to the cysteine probably as a chain of the ECA molecules. Kandror et al. [27] has shown that thiols containing an amino group can cause polymerization of cyanoacrylates.

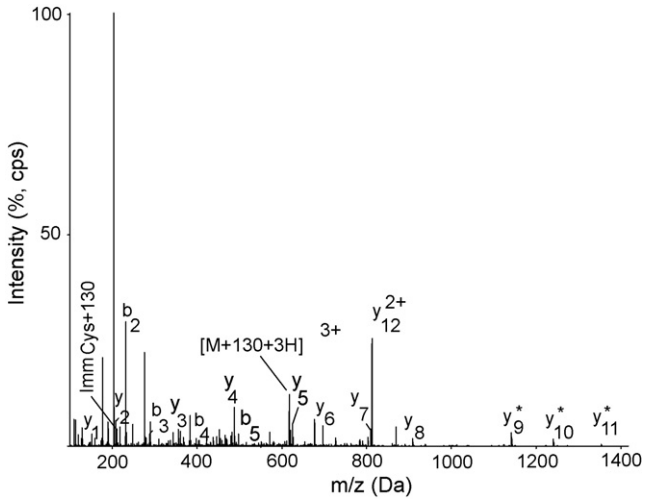

Fig. 4. Product ion spectra of the ion $[M+130+3H]^{3+}$ at m/z 617.40 corresponding to the tryptic HEMA adducted peptide LLGNVLVC*VLAHHFGK (β 105–120). The adduct is located on Cys^{112}. The * denotes the site of modification. The analysis was performed using nano electro spray/hybrid quadrupole time-of-flight mass spectrometry.

The ions found in the Hb–MMA conjugate were ValCys-MMA (m/z 321.17) and HisCys-MMA (m/z 359.19). The dipeptid HisCys corresponds to either Cys^{93} and His^{94} of the β-chain or Cys^{104} and His^{103} of the α-chain. Both ValCys-MMA and HisCys-MMA showed a fragment at m/z 222.10, corresponding to the adducted cysteine. Thus, cysteine was identified as the binding amino acid.

The ions found in the Hb–HEMA conjugate was ValCys-HEMA (m/z 351.20), and HisCys-HEMA (m/z 389.21). Both ValCys-HEMA and HisCys-HEMA showed a fragment at m/z 252.11, corresponding to the adducted cysteine. Thus, cysteine was identified as the binding amino acid. HisCys-HEMA was not observed in the computer analysis, but was identified using nanoES-QqTOF since HEMA seem to react in a similar way as MMA.

3.5. Test of reactivity between N-acetyl-L-lysine and ECA, MMA and HEMA

Analysis of the reaction product of N-acetyl-L-lysine and ECA, MMA or HEMA was performed using LC/MS/MS in MS scan mode. No N-acetyl-L-lysine adducts were observed at the physiological pH used in our study. In addition, using nanoES-QqTOF we did not find any adducts on the N-terminal valines of Hb, which is known to form adducts with electrophiles [21,23,28]. This is in contrast to Gerberick et al. [15] who found adducts between ethyl acrylate or 2-hydroxyethyl acrylate and the amino group of lysine. On the other hand, they used a much higher pH at 10.2 which could have influenced the adduct formation considerably.

3.6. NanoES-QqTOF analysis of full-length Hb–ECA, Hb–MMA, and Hb–HEMA conjugate

Un-digested conjugates from each acrylate were analyzed with nanoES-QqTOF in order to determine the number of adducts formed for each conjugate. The reconstructed spectra of the Hb–ECA conjugate showed a complex adduct formation. The α-chain (m/z 15126 Da) was observed followed by several peaks. One peak, with an additional mass of 97 Da, suggested binding of one ECA that had lost an ethyl group. A second peak with an additional mass of 125 Da suggested binding of one ECA, a third peak with an additional mass of 222 Da suggested $\alpha + 1ECA^{97\,Da} + 1ECA^{125\,Da}$, and a fourth peak with an additional mass of 250 Da suggested $\alpha + 2ECA^{125\,Da}$. There is only one cysteine in the α-chain. This further supports the results from nanoLC-QqTOF analysis of pronase E digested conjugates, where the ECA polymerized during the synthesis of the conjugate. The same pattern of adduct formation was observed for the β-chain (m/z 15867 Da), where additional masses of 97, 125, 222, and 250 Da were observed. In the β-chain there are two cysteines but in the results from the nanoES-QqTOF only the Cys^{112} was found adducted.

The reconstructed spectra of the full-length Hb–MMA conjugate showed the α-chain followed by a peak with an additional mass

Table 1
Adducted Hb–HEMA, Hb–MMA and Hb–ECA tryptic peptides analyzed using offline nano electro spray/hybrid quadrupole time-of-flight mass spectrometry. Only the cysteine moieties react with the acrylates.

Peptide	Sequence	m/z	z	MSight[a]	MoDetect[a]
α 100–127 + ECA 250	LLSHC*LLVTLAAHLPAEFTPAVHASLDK	644.35	5	No	Yes
β 105–120 + ECA 222	LLGNVLVC*VLAHHFGK	648.04	3	Yes	Yes
		486.30	4	Yes	Yes
α 100–127 + MMA	LLSHC*LLVTLAAHLPAEFTPAVHASLDK	767.69	4	Yes	Yes
		614.30	5	Yes	Yes
β 83–95 + MMA	GTFATLSELHC*DK	761.40	2	Yes	Yes
		507.95	3	Yes	Yes
β 83–104 + MMA	GTFATLSELHC*DKLHVDPENFR	658.10	4	Yes	Yes
		526.70	5	Yes	Yes
β 105–120 + MMA	LLGNVLVC*VLAHHFGK	607.30	3	Yes	Yes
		455.80	4	Yes	Yes
α 100–127 + HEMA	LLSHC*LLVTLAAHLPAEFTPAVHASLDK	775.21	4	Yes	Yes
		620.35	5	Yes	Yes
β 83–95 + HEMA	GTFATLSELHC*DK	776.39	2	No	Yes
		517.95	3	Yes	Yes
β 83–104 + HEMA	GTFATLSELHC*DKLHVDPENFR	665.60	4	Yes	Yes
		532.60	5	Yes	Yes
β 105–120 + HEMA	LLGNVLVC*VLAHHFGK	617.40	3	Yes	Yes
		463.20	4	Yes	Yes

*The adducted amino acid.
[a] Yes = detected; no = not detected.

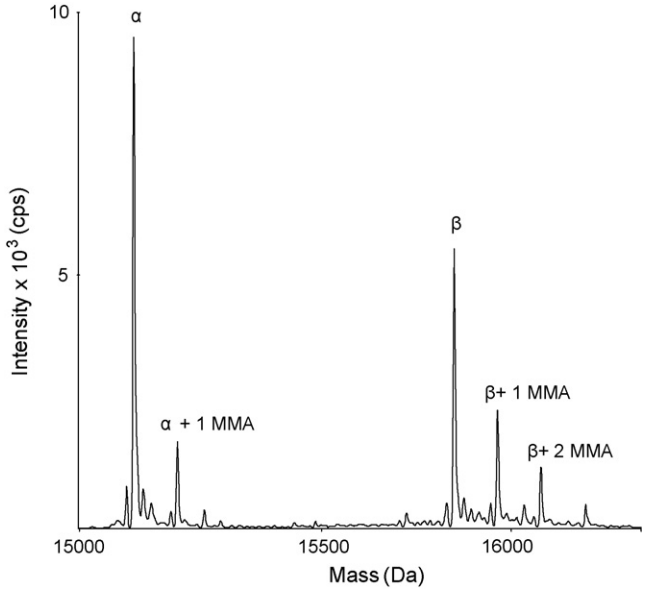

Fig. 5. Nano electro spray/hybrid quadrupole time-of-flight mass spectrometric analysis of the full-length conjugate of Hb–MMA showing the reconstructed mass spectra of the Hb–MMA conjugate.

of 100 Da suggesting α-chain + 1 MMA. The peak for the β-chain was followed by two peaks each peak adding 100 Da suggesting β-chain + 2MMA (Fig. 5).

The full-length reconstructed spectra of Hb–HEMA showed the α-chain followed by a peak with an additional mass of 130 Da suggesting α + 1 HEMA. The peak for the β-chain was followed by two peaks; each peak adding 130 Da suggesting β + 2 HEMA. This is also in line with the results from the nanoES-QqTOF that the acrylates are bound to cysteines.

Table 3
SRM transitions for pronase E digested Hb adducted with HEMA, MMA and ECA using LC/MS/MS.

Fragment	Transitions (*m*/*z*) Q1/Q3	Q3-ion	DP[a]	CE[b]
ValCys-ECA$^{222\,Da}$	443.3/344.3	Cys-ECA$^{222\,Da}$	40	25
	443.3/231.0		40	25
ValCys-MMA	321.2/221.9	Cys-MMA	40	21
	321.2/205.2		40	26
ValCys-HEMA	351.3/252.2	Cys-HEMA	36	34
	351.3/113.3		50	36

[a] Declustering potential.
[b] Collision energy.

3.7. LC/MS/MS of adducted peptides using SRM

Parameters of the LC/MS/MS methods in the SRM mode were optimized using trypsin digested conjugates from Hb–ECA, Hb–MMA, and Hb–Hema. Methods for all identified adducted peptides were optimized. The DP was set at 30 V for all peptides except β 105–120 + ECA$^{125\,Da}$ + ECA$^{97\,Da}$ where DP was set to 40 V. The chosen CE are described in Table 2. The peptide α 100–127 + ECA$^{250\,Da}$ was not detected. The adducted peptide, β 105–120, showed the best response, with the lowest signal/noise for all three conjugates (Fig. 6). Standard curves of trypsin digested conjugates in the range 2.1–66.7 μg/ml were linear with correlation coefficients above 0.99 using linear regression. The adducted peptide, β 105–120 could be detected down to 2.1 μg/ml. However, it must be emphasized that the concentrations used here are calculated from the amount of adducted protein. Thus, the actual concentration of the adducted peptide is probably much lower.

LC/MS/MS analysis of pronase E digested conjugates from Hb–ECA, Hb–MMA, and Hb–HEMA using SRM was performed only for the ValCys adducts (Fig. 7) since the abundance for the HisCys adduct was very low. The transitions, DP, and CE used are described in Table 3. Calibration curves of pronase E digested conjugates

Table 2
SRM transitions for trypsinated Hb adducted with HEMA, MMA and ECA using LC/MS/MS. Only the cysteine moieties react with the acrylates.

Peptide	*z*	Sequence	Transitions (*m*/*z*) Q1/Q3	Q3-ion	CE[a]
α 100–127 + ECA 250	5	LLSHC*LLVTLAAHLPAEFTPAVHASLDK	644.4/N.D.[b]	N.D.	N.D.
β 105–120 + ECA 222	3	LLGNVLVC*VLAHHFGK	648.0/227.2	b_2	26
			648.0/648.0	Pre.[c]	9
α 100–127 + MMA	4	LLSHC*LLVTLAAHLPAEFTPAVHASLDK	767.7/767.7	Pre.	35
			767.7/469.4	y_9^{2+}	35
β 83–95 + MMA	2	GTFATLSELHC*DK	761.3/465.4	y_3*	35
			761.3/1145.7	y_9	35
β 83–104 + MMA	4	GTFATLSELHC*DKLHVDPENFR	658.1/752.4	b_6	35
			658.1/662.5	y_5	35
β 105–120 + MMA	4	LLGNVLVC*VLAHHFGK	455.7/488.2	y_4	35
			455.7/176.3	Imm.[d]	35
α 100–127 + HEMA	4	LLSHC*LLVTLAAHLPAEFTPAVHASLDK	775.2/775.2	Pre.	35
			775.2/937.5	y_9	35
β 83–95 + HEMA	2	GTFATLSELHC*DK	776.4/495.2	y_3*	35
			776.4/961.8	y_7	35
β 83–104 + HEMA	4	GTFATLSELHC*DKLHVDPENFR	665.6/834.4	y_6	35
			665.6/661.3	y_{15}^{3+}	35
β 105–120 + HEMA	3	LLGNVLVC*VLAHHFGK	617.3/812.8	y_{14}^{2+}	35
			617.3/1142.1	y_9*	35

*The adducted amino acid.
[a] Collision energy.
[b] Not detected.
[c] Precursor ion.
[d] Immonium ion.

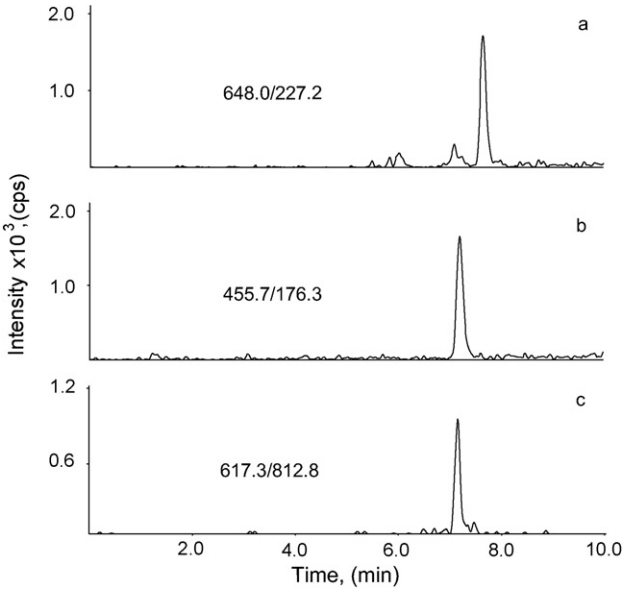

Fig. 6. LC/MS/MS analysis of 8 μg/ml tryptic digests of conjugated Hb. Extracted ion chromatogram of transitions at (a) 648.0 → 227.2 correspond to β 105–120 + ECA$^{125\,Da}$ + ECA$^{97\,Da}$ (b) 455.7 → 176.3 correspond to β 105–120 + MMA and (c) 617.3 → 812.8 correspond to β 105–120 + HEMA.

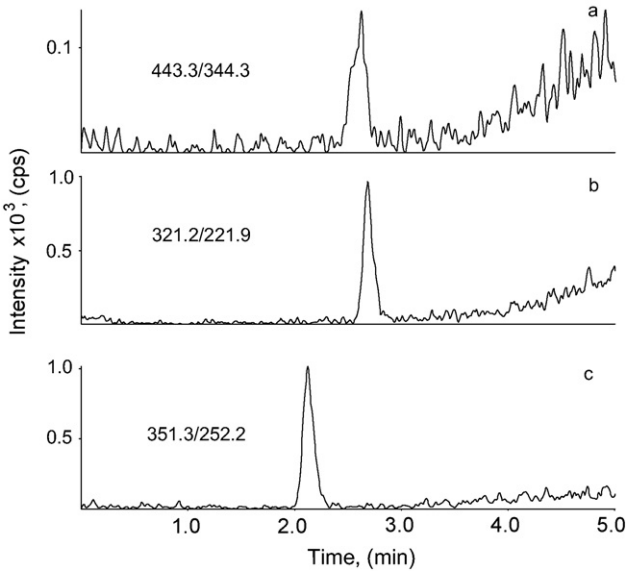

Fig. 7. LC/MS/MS analysis of 1 μg/ml pronase E digests of conjugated Hb. Extracted ion chromatogram of transitions at (a) 443.3 → 344.3 correspond to ValCys-ECA (b) 321.2 → 222.9 correspond to ValCys-MMA and (c) 351.3 → 252.2 correspond to ValCys-HEMA.

in the range 0.1–66.7 μg/ml were linear with correlation coefficients above 0.99 using linear regression. ValCys-ECA was observed down to 0.5 μg/ml. Both ValCys-MMA and ValCys-HEMA could be detected down to 0.1 μg/ml. However, it should be emphasized that the concentrations are not the concentration of the ValCys-conjugates but based on the weighted amount of conjugate. The limit of detection was defined as a peak three times higher than the noise.

3.8. Biomarkers of exposure

There are no specific biomarkers available to measure the exposure of acrylates. It has been shown in this work that adducted tryptic peptides as well as the ValCys-adducted dipeptide when digested with pronase E can be analyzed with SRM using LC/MS/MS. Such peptides have previously been used for biological monitoring. Jeppsson et al. [29] used tryptic albumin peptides adducted with methylhexahydrophthalic anhydride to investigate the exposure of workers using LC/MS/MS in the SRM mode. In addition, Noort et al. [30] showed that pronase E digest of albumin could be used as a biomarker of exposure to mustard gas. In that study the adducted cysteine was cleaved into a tripeptide and then analyzed by LC/MS/MS in the SRM mode. Thus, the adducted peptides and dipeptide identified in the present study has the potential to become specific biomarkers for exposure of ECA, MMA, and HEMA after further evaluation. As a next step in such an evaluation the adducted peptides should be synthesized and purified and the sensitivity of the peptide can then be evaluated. Since the acrylates preferably binds to cysteines such synthesis should be easy to perform. In addition, Hb from exposed workers should be analyzed to determine the levels of adducts in these subjects. If the adduct levels are found to be too low to be detected, there are ways to solve this problem; e.g., Georgieva et al. [31] and Johannesson et al. [32] have shown that adducted peptide can be purified using immunoaffinity chromatography. It is also possible worth evaluating if peptides from human serum albumin or other high abundant plasma proteins can be analyzed.

4. Conclusions

This study showed that acrylates bind covalently to cysteines in Hb. ECA binds covalently to the cysteines at the 104 position in the α and the position 112 in the β-chains in Hb. MMA and HEMA bound to all the cysteines in both chains, Cys^{104} in the α-chain and Cys^{93} and 112 in the β-chain. The full-length spectra of in undigested Hb confirmed this biding pattern. There was no reaction with N-acetyl-L-lysine at physiological pH. We have shown that adducted peptides can be measured with LC/MS/MS using SRM. These peptides may be used for biological monitoring of exposure to ECA, MMA and HEMA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.04.026.

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