Acetaldehyde adducts of human hemoglobin: Identification of modified hemoglobin N-terminal peptides in blood samples by LC/TOF-MS

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Abstract

Background: Excessive alcohol consumption can cause a variety of long-term sequelae like damages to the nervous system and liver or cancer. For the latter, Acetaldehyd is regarded as carcinogenic noxa. While Acetaldehyde adducts of hemoglobin have been considered as potential biochemical markers of ethanol exposure a chemical identification in human blood samples has not been achieved.

Method: Hemoglobin and 56 blood samples were analyzed after trypsin digestion using LC/TOF-MS (time-of-flight mass spectrometer).

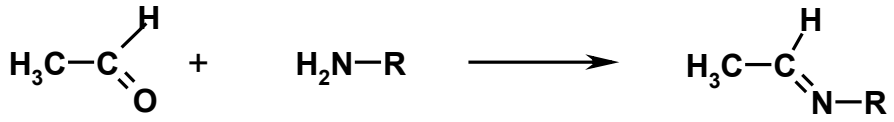
Results: After incubation of hemoglobin in-vitro with increasing concentrations of acetaldehyde followed by tryptic digestion of the incubate different modifications could be identified by their accurate mass and retention time shift as well as in post mortem blood. Acetaldehyde is covalently bound to N-terminal peptides on both major globin chains (Hbα and Hbβ). Using forensic samples from 40 deceased persons exhibiting different ethanol levels (between 0 and 4.1 g/l) marked difference in abundance of multiple acetaldehyde modified peptide fragments was found in the tryptic digest of the hemolysates linked to acetaldehyde and not to post-mortal alteration.

Conclusion: Exposition to acetaldehyde, e.g. in heavy ethanol abusers, may lead to a modification of hemoglobin chains. Abundances of modified tryptic peptides were higher in samples with elevated blood alcohol concentration and are therefore considered to be a marker at excessive ethanol consumption.

1. Introduction

Effective treatment of ethanol abuse is often hampered by the lack of specific markers suitable to detect ethanol consumption over long time periods. Acetaldehyde as first oxidative metabolite of ethanol binds in vivo to a wide variety of structures such as plasma proteins like albumin [5], CYP450 [2], low density lipoproteins [22], ribonuclease [15], nucleosides [6] and to hemoglobin (Hb) [20]. The latter, e.g. acetaldehyde adducts of hemoglobin, is regarded as a potential marker of ethanol consumption [1, 8, 10, 11, 24]. Hemoglobin can be easily obtained from blood samples and due to its long half-life of approximately 3 months, hemoglobin modified by reactive molecules may accumulate. It is particularly useful as target molecule for monitoring post-translational modifications [7].

Several studies have been performed on acetaldehyde hemoglobin adducts using immunoassay [13], radiolabelling [16,17,25], liquid chromatography [25], cation exchange chromatography [10, 14], NMR [4], Raman spectroscopy [4] or mass spectrometry [3, 28]. Acetaldehyde was expected to form stable adducts with amino or hydroxyl groups. Whereas Tuma et al. [27] considered lysine residues to be the exclusive binding sites San George and Hoberman [21] found that acetaldehyde also binds to other amino acids such as tyrosine, valine, glucosylvaline and glucosyllysine and that acetaldehyde residues may be exchanged among peptides. Structural studies on a pentalysine model peptide incubated with acetaldehyde using FAB/MS indicated the formation of a Schiff´s base or an imidazolidinone moiety under nonreduced conditions [3]. An acetaldehyde adduct of the synthetic N-terminal beta-chain octapeptide VHLTPEEK of hemoglobin was observed by Braun and co-workers [4] by using ESI-MS, NMR spectroscopy and Raman spectroscopy and was considered to be a potential biochemical marker for ethanol consumption.



Acetaldehyde Aminofunction Schiff base

Apart from in-vitro studies, early approaches to obtain evidence for increased adduct levels in human subjects were not successful [12, 26]. Studies by Niemela et al. [18,19] using antibodies against acetaldehyde-modified protein epitopes, indicated a correlation between ethanol consumption habits and elevated acetaldehyde adducts. Measurement of a Hb fraction named $HbA1_{ach}$ identified by Sillanaukee and co-workers [23] was interfered by glycosylation phenomena. Later studies suggested similar evidence when compared with other ethanol markers like MCV, GGT, AST and ALT [9]. Itälä et al. [14] developed a cation exchange chromatographic procedure for the separation of globin chains, which yielded three fractions considered to represent acetaldehyde-Hb adducts. One named $HbA1_{ach3}$, was reported to be free of interference with acetylated and glycated Hb.

Analytical techniques have considerably improved such as liquid chromatography with a new online time-of-flight (TOF-MS) mass spectrometric detection device and provided a new opportunity to detect Hb-acetaldehyde adducts in-vivo with high sensitivity. For this purpose, procedures for the analysis of hemoglobin chains and of peptides obtained after tryptic digestion were developed and evaluated using in-vitro incubation of Hb with acetaldehyde and were consecutively used for the analysis of authentic human hemoglobin.

2. Material and Methods

2.1 Instrumentation

The HPLC system (Agilent 1100 series) used was equipped with degasser, binary pump, and column oven. Sample injection was automatically performed using a 54-well plate sampler onto a Polaris-C18-Ether column (100 x 2.0 mm, 3 µm particle size) from Varian (Darmstadt, Germany) connected to a time-of-flight mass spectrometer (TOF-MS) from Agilent Technologies (Waldbronn, Germany). The LC/TOF-MS interface was an electrospray ion source (ESI) equipped with a dual-sprayer mechanism that allows constant injection of reference substances (purine at 121.050873 Da and HP-921 at 922.009798 Da) for mass shift correction in every spectrum acquired, thus providing mass accuracies in the range of ±3 ppm. ESI source and TOF-MS parameters were set according to the recommendations of the supplier except for nebulizer pressure (50 psig), capillary voltage (4000V) and drying gas flow (12 l/min at 350°C), which were set according to recommendations for a flow rate of 0.4 ml/min. The fragmentor voltage was set to 180 V according to preliminary optimization studies.

Agilent TOF Software Version A 02.00 was used for data acquisition. Data evaluation was performed using Analyst QS 1.1 software (Applied Biosystems/MDS Sciex, Concord, Canada). Deconvolution of protein data and simulation of tryptic digests was performed with Agilent BioConfirm Version A 02.00 software in combination with accurate peptide masses provided by the ExPasy Proteomics Server (www.expasy.org). The Molecular Weight Calculator Version 6.73 software was used for peptide sequence fragmentation modeling along with simulation of hypothetical acetaldehyde adducts and isotopic distribution modeling (http://ncrr.pnl.gov/software/). Mass Hunter and Molecular Feature Extractor as integrated in the Agilent TOF Software were used for data reduction according to the manufacturer's recommendations. Reduced data was evaluated with Agilent Mass Profiler MFE 13 software. For identification of modified and unmodified peptides an error of the accurate mass of ±5 ppm was accepted and a retention time window of ±0.2 min in reference to the analysis of a reference digest of hemoglobin and synthetically generated adducts respectively. Integration was carried out using standard software parameters.

2.2 Reagents

For all purposes HPLC-grade water was used from Waters (Eschborn, Germany); HPLC-grade acetonitrile was from Baker (Netherlands); TPCK-treated trypsin (40 U/mg), formic acid (FA), acetaldehyde and all other reagents were of analytical grade and from Merck (Darmstadt, Germany).

2.3 Biological samples

Blood samples from sixteen children between 1 and 9 years old were analyzed since it could be expected that the children had not been exposed to ethanol previously. These samples, originally submitted for therapeutic drug monitoring of antiepileptic drugs were analyzed after approval by the local ethics committee.

Blood samples from 9 female and 31 male deceased persons between 6 days and 80 years old were obtained during section in the Institute of Forensic Medicine (Center of Legal Medicine, Frankfurt, Germany) and stored at 4°C for toxicological analysis. These post-mortem samples were selected in retrospect. Criteria were sample availability and blood alcohol concentration (BAC) ranging between 1.1 g/L and 4.1 g/L (n=24) in one group and not detectable (n=16) in the other group considered as negative control.

2.4 Hemolysis of blood

Aliquots (about 1ml) of blood or erythrocyte concentrate were washed twice with equal volumes of physiological saline and centrifuged at 2,000 g for 10 minutes. The supernatants were discarded and the concentrated erythrocytes were lysed by adding one aliquot of distilled water and incubated at room temperature for 30 minutes followed by centrifugation for 10 minutes at 16,000 g to remove cell debris. The hemoglobin content in the supernatant hemolysate was determined by an automated photometric method (Haemoxymeter, Radiometer, Copenhagen, Denmark) and the hemolysate was divided into aliquots for analysis.

2.5 Analysis of hemoglobin chains

A volume of hemolysate containing 8 mg (123 nmol) hemoglobin was mixed with 75 µl of trifluoroethanol and 75 µl of 30 mM NH_4HCO_3 (pH 8.0) and incubated for 45 minutes at 60°C for protein denaturation. The mixture was diluted tenfold with 1.8 mL of 30 mM NH_4HCO_3 (pH 8.0). 100 µl of hemolysate containing approximately 0.4 mg hemoglobin were mixed with an equal volume of acetonitrile/water (74:26, v/v) containing 10% FA and centrifuged at 16,000 g for 10 minutes. Of the supernatant 0.5 µl were analyzed by LC/TOF-MS using a linear gradient with 5% FA in water as solvent A and acetonitrile as solvent B. The gradient increased from 37% to 39% solvent B in 10 minutes followed by an additional washing step with 100% acetonitrile for 3 minutes. The flow rate was 0.4 ml/min and the column was operated at 50 °C.

2.6 Analysis of peptides after tryptic digestion of blood hemolysate

A volume of hemolysate containing 8 mg (usually 50 µl) hemoglobin was mixed with 75 µl of trifluoroethanol and 75 µl of 30 mM NH_4HCO_3 (pH 8.0) and incubated for 45 minutes at 60°C for protein denaturation. The mixture was diluted tenfold with 1.8 mL of 30 mM NH_4HCO_3 (pH 8.0) to prevent inactivation

of the cleavage enzymes. Trypsin solution (160 µl of 1 mg/ml in buffer) was added, incubated for 24 hours at 37°C, and the incubate was lyophilized. The dry residue was dissolved in 200 µl water and centrifuged at 2,000 g for 10 minutes to remove undigested cell debris prior to analysis of 2 µl by LC/TOF-MS using a linear gradient with 0.1% FA in water as solvent A and acetonitrile as solvent B. The gradient increased from 0% to 40% solvent B in 24 minutes followed by an additional washing step with 100% acetonitrile for 5 minutes. The flow rate was 0.4 ml/min and the column was operated at 60 °C.

For data analysis the primary sequence of the hemoglobin chains were theoretically cleaved by trypsin using Agilent BioConfirm software and the accurate masses of the resulting peptides were calculated using Molecular Weight Calculator software. For the identification of acetaldehyde modified peptides the peptide masses were increased by 26.0157 (addition of CH_3CHO and loss of H_2O).

2.7 Preparation of acetaldehyde-modified hemoglobin

Hemolysate was incubated in 1.5 ml reaction tubes with acetaldehyde for 24 hours at 37°C. The following concentrations were used: 5, 10, 20, 50, 100, and 500 mM. After incubation, samples were processed for analysis of hemoglobin chains or peptides obtained after tryptic digestion, respectively.

3. Results and discussion

Acetaldehyde, the toxic metabolite of ethanol metabolism, has been found to react with several proteins. One of them is hemoglobin, which has a long half-life and therefore modified molecules may accumulate after exposure to reactive chemicals. This makes hemoglobin suitable as a long-term biomarker. Efforts have been made to detect acetaldehyde modified hemoglobin as marker of ethanol exposure. Although immunoassay [19], cation exchange chromatography [24] and MALDI-TOF method [28] indicated the presence of such adducts, identification of the chemical binding sites has not been achieved. The aim of the present study was to evaluate analytical procedures for the detection of acetaldehyde adducts of hemoglobin using liquid chromatography coupled to time-of-flight mass spectrometry.

3.1 Analysis of hemoglobin chains

Although the sensitivity of the analysis procedure for uncleaved hemoglobin chains seems to be appropriate for the assay of the major post-translational hemoglobin modifications, in none of the post-mortem blood samples acetaldehyde adducts could be detected.

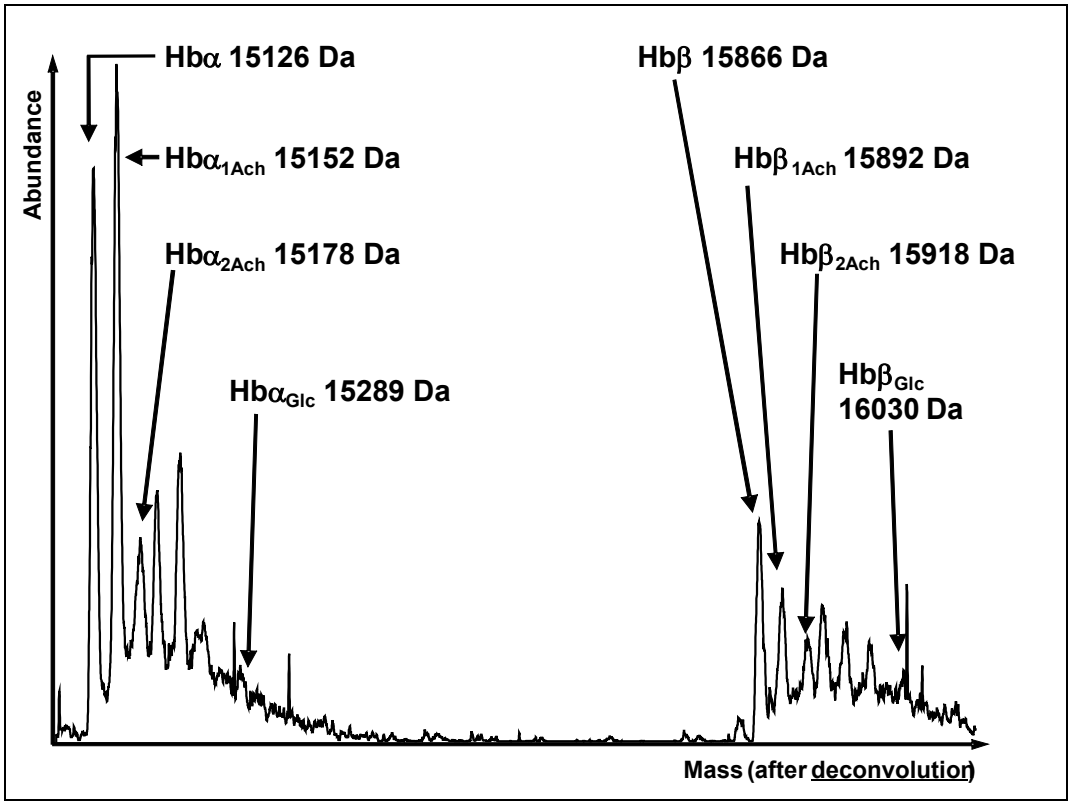


Abb. 1 Deconvoluted mass spectrum of acetaldehyde incubated human erythrocyte concentrate hemolysate.

3.2 Analysis of trypsin digested hemoglobin

Peptide fragments of hemoglobin modified with acetaldehyde should be detectable with much higher sensitivity than hemoglobin chains, since acetaldehyde modification leads to marked polarity changes of the peptide fragments involved and should enable their chromatographic separation. Therefore hemolysates were digested with trypsin and the digest was analyzed without further purification. Deviation from the accurate peptide fragment masses of less than 5 ppm and a retention time of ±0.2 min in comparison to a digest of hemoglobin reference material were criteria for peptide identification.

In order to identify acetaldehyde binding sites on the hemoglobin chains, RBC hemolysate was incubated with increasing concentrations of acetaldehyde (0.05 to 500 mM for 24 h at 37°C) followed by trypsin digestion. The resulting peptide mixtures were screened for acetaldehyde modified peptides. For both N-terminal peptide fragments modifications could be observed. The abundances increased with higher acetaldehyde concentrations and their retention times were always higher than those of the corresponding non-modified peptides, which is in accordance with a reduced polarity due to the alkylation. Modified peptides of equal accurate masses eluting at different retention times indicate the existence of

multiple binding sites corresponding to the results observed in hemoglobin chain analysis where also multiple adducts were found after incubation with acetaldehyde. This confirms previous findings indicating that acetaldehyde can bind to different amino acids such as lysine, tyrosine and the N-terminal valine [3, 21]. These studies focused on the detection of an acetaldehyde modification at the N-terminal octapeptide of the beta globin chain [4], but this has never been detected in authentic blood samples [28].

3.3 Analysis of authentic blood samples

In the present study blood samples from fourteen children between 1 and 9 years old were analyzed, since it could be expected that the children had not been exposed at all to ethanol previously. In consequence no altered hemoglobin chains should be present in those samples or only in trace amounts caused by endogenous acetaldehyde. Additionally post-mortem samples from forty persons deceased at ages between 6 days and 80 years old were analyzed representing sixteen samples (4 female, 12 male) with BAC 0 g/L which were to be compared to twenty-four samples (5 female, 19 male) with BAC between 1.1 g/L and 4.1 g/L.

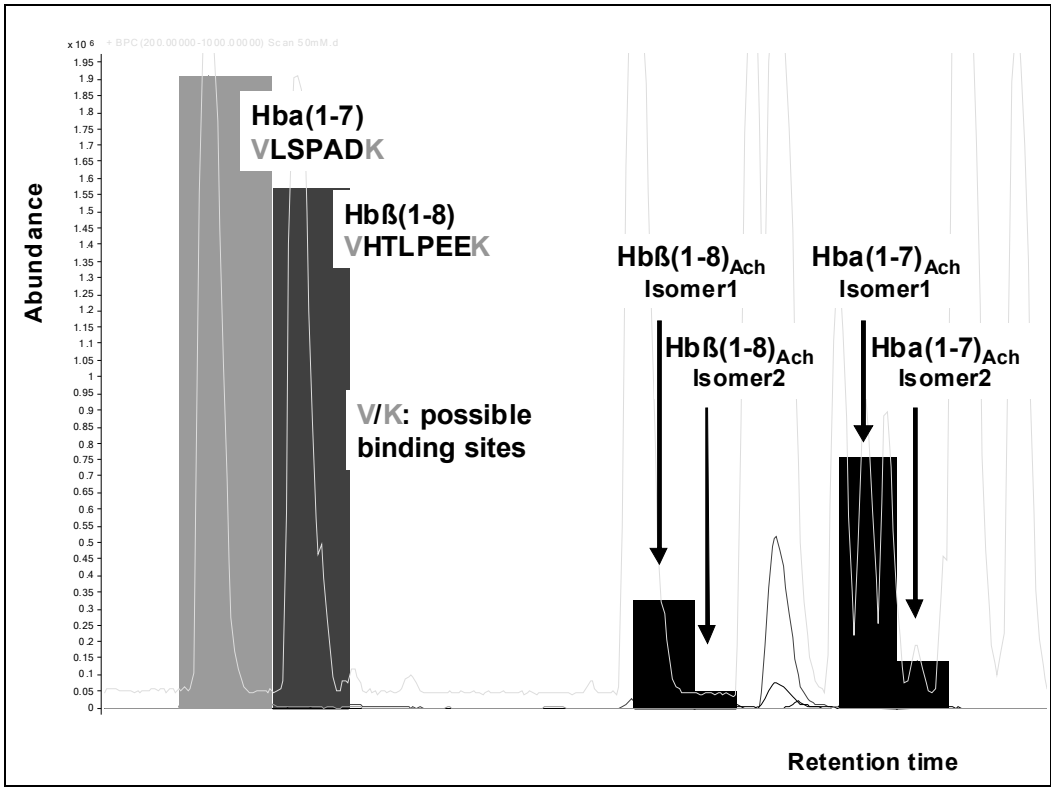


Abb. 2 Extracted ion chromatogram of unmodified and respective acetaldehyde modified (isomeric) N-terminal peptides of both hemoglogin chains.

The detection of acetaldehyde modified hemoglobin in-vivo had been successful only by using immunologic and electrophoretic methods and in a recent study analyzing minor hemoglobins in authentic blood samples using MALDI-TOF [28], the tryptic digest of a globin chain with a mass of 15.894 Da, which is consistent with an acetaldehyde adduct of the β-chain, exhibited no acetaldehyde modification at the N-terminal peptide indicating other sites than the N-terminus as binding site for acetaldehyde in-vivo.

By analysis of the authentic samples in the present study, essentially the same results as for the reference materials (hemoglobin substance and hemolysate of RBC concentrate) were obtained. Two modified fragments of the N-terminal peptides of both hemoglobin chains, the alpha globin chain terminus (VLSPADK, $Hb\alpha(1\text{-}7)_{Ach1}$ /$Hb\alpha\ (1\text{-}7)_{Ach2}$) and the beta globin chain terminus (VHLTPEEK, $Hb\beta(1\text{-}8)_{Ach1}$ /$Hb\beta(1\text{-}8)_{Ach2}$), could be identified for the first time in the authentic samples. Area ratios of modified peptides vs. corresponding unmodified peptide were compared between samples from fourteen children and blood samples from sixteen corpses not intoxicated by alcohol at the time of death as control group as well as to blood samples from twenty-four corpses with BAC above 1.1 g/L at the time of death using chi-square test. Comparing samples from deceased non-intoxicated with children significant differences in the area ratios of modified

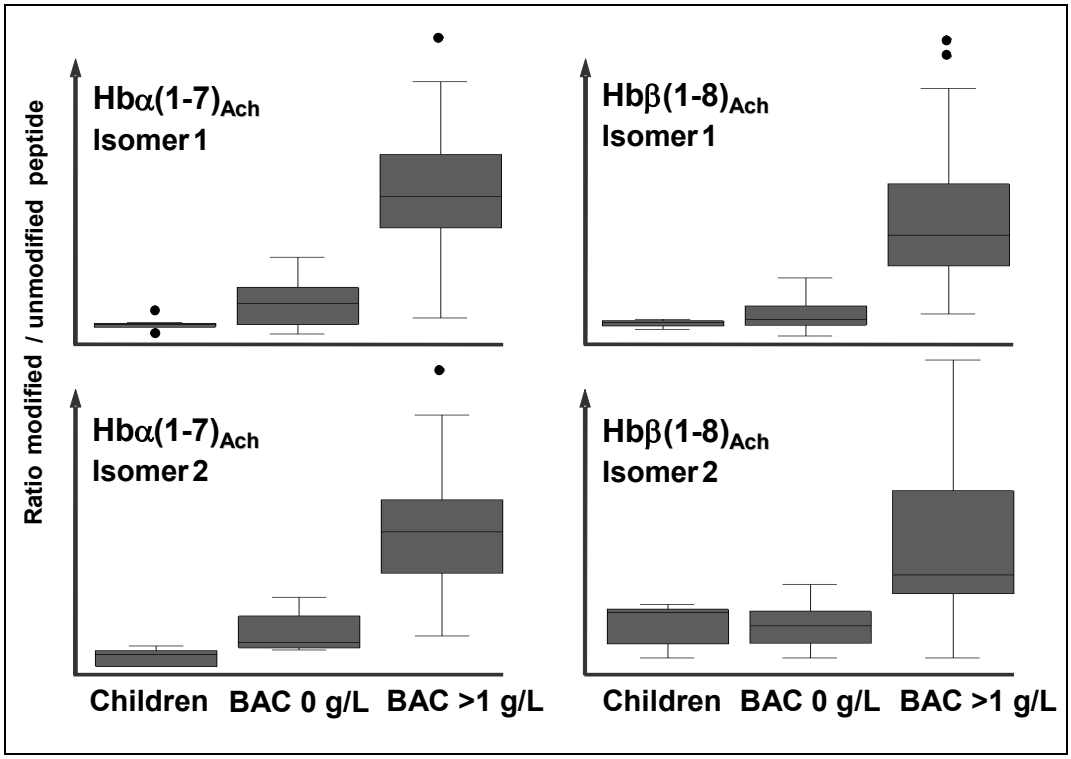


Abb. 3 Box plots of the distribution of the area ratios (modified vs. unmodified peptides) in the three groups studied.

peptides were observed ($p < 0.05$) except for the peptide $Hb\beta(1\text{-}8)_{Ach1}$ while abundances in the samples of the post-mortem group with BAC>1 g/L were highly significantly elevated ($p<0.001$) in comparison to the other two groups. Decomposition of the samples during time of storage had no influence on modified peptides since all post-mortem samples were stored over the same period of time ranging from 3 to 18.5 months for samples with high blood alcohol levels and 4 to 18.5 months for the control group respectively (data not shown).

Therefore the different ratios of modified vs. unmodified hemoglobin was supposed to be acetaldehyde-induced and linked to ethanol exposure in consequence. Hypothetical long-term accumulation of acetaldehyde adducts and studies on the kinetics of the post-translational modifications will be performed in the future. Nevertheless these acetaldehyde modified peptides seem to be a promising biochemical marker to detect excess ethanol consumption.

4. Conclusion

Continued exposition to acetaldehyde, e.g. in heavy ethanol abusers, may lead to an accumulation of acetaldehyde modified hemoglobin. In authentic samples acetaldehyde adducts could be detected after trypsin digestion but not by analysis of total globin chains. Comparison of samples obtained from living children as well as from non-intoxicated and ethanol-intoxicated corpses showed significant differences in abundances of modified hemoglobin. Modified tryptic peptides were significantly elevated in samples with high blood alcohol concentrations indicating a correlation between alcohol intake and abundance of acetaldehyde adducts. Therefore such acetaldehyde modified peptides might be markers for excessive ethanol consumption.

5. References

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