Chemical Research in Toxicology

Article

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LC−MS/MS Screening Strategy for Unknown Adducts to N-Terminal Valine in Hemoglobin Applied to Smokers and Nonsmokers

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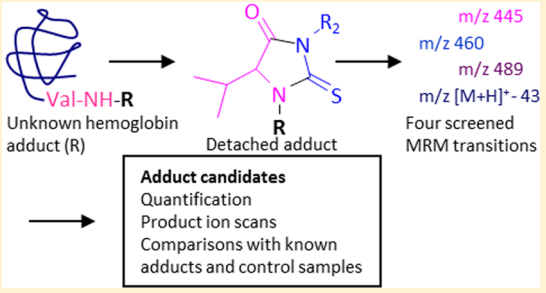
Supporting Information

ABSTRACT: Electrophilically reactive compounds have the ability to form adducts with nucleophilic sites in DNA and proteins, constituting a risk for toxic effects. Mass spectrometric detection of adducts to N-terminal valine in hemoglobin (Hb) after detachment by modified Edman degradation procedures is one approach for *in vivo* monitoring of exposure to electrophilic compounds/metabolites. So far, applications have been limited to one or a few selected reactive species, such as acrylamide and its metabolite glycidamide. This article presents a novel screening strategy for unknown Hb adducts to be used as a basis for an adductomic approach. The method is based on a modified Edman procedure, FIRE, specifically developed for LC−MS/MS analysis of N-terminal valine adducts in Hb detached as fluorescein thiohydantoin (FTH) derivatives. The aim is to detect and identify *a priori* unknown Hb adducts in human blood samples. Screening of valine adducts was performed by stepwise scanning of precursor ions in small mass increments, monitoring four fragments common for the FTH derivative of valine with different N-substitutions in the multiple-reaction mode, covering a mass range of 135 Da (m/z 503−638). Samples from six smokers and six nonsmokers were analyzed. Control experiments were performed to compare these results with known adducts and to check for artifactual formation of adducts. In all samples of smokers and nonsmokers, seven adducts were identified, of which six have previously been studied. Nineteen unknown adducts were observed, and 14 of those exhibited fragmentation patterns similar to earlier studied FTH derivatives of adducts to valine. Identification of the unknown adducts will be the focus of future work. The presented methodology is a promising screening tool using Hb adducts to indicate exposure to potentially toxic electrophilic compounds and metabolites.



INTRODUCTION

Electrophilic compounds/metabolites are known to form adducts with nucleophilic sites in DNA and proteins and thereby constitute a risk for toxic effects. Many of those reactive substances are practically impossible to measure as free compounds in tissue samples. Therefore, methods to measure their stable reaction products (adducts) with biomacromolecules have been developed.[1,2] Initially, such methods were aimed at the *in vivo* monitoring of occupational exposure.[3] Measurements of adducts with DNA or blood proteins also have disclosed exposure to electrophiles in the general population, in smokers as well as in nonsmokers. A number of studies of adducts to hemoglobin (Hb) and serum albumin (SA) applying mass spectrometric (MS) analysis have identified such "background" exposures to compounds that are genotoxic and classified as carcinogens in animals or humans. Examples are ethylene oxide from natural endogenous formation[4] and tobacco smoking,[5] acrylamide from food processing[6] and tobacco smoking,[7] metabolites of benzene, probably from endogenous sources,[8,9] tobacco-specific nitrosamine adduct,[10] and aromatic amines,[11] e.g., 4-aminobiphenyl with increased levels from tobacco smoking.[12]

The detection of background exposure through adducts has stimulated the development of the adductomics concept, aiming at the identification of unknown exposure to electrophilic compounds/metabolites in the general population in order to search for causative agents in chronic diseases.[13,14] The adductomics approach aims at studying all possible adducts (within a specified mass range) to specific nucleophilic sites in a certain biomacromolecule. So far, there are promising adductomic studies of both DNA adducts (reviewed by Balbo et al.[15]) and human serum albumin (HSA) adducts.[14,16] Determination of blood protein adducts has the advantages of easy access to a large amount of sample, accumulation with known kinetics of stable adducts, and established sensitive MS methodologies for their analysis.

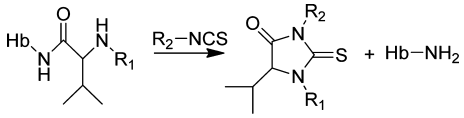
One reactive nucleophilic site in Hb is the N-terminal valine (Val), present in both the α- and β-chains in adult human Hb as well as in Hb from mouse and rat. Methods for determination of Hb adducts have been developed, based on modified Edman degradation procedures, with specific detachment and isolation of N-substituted N-terminal Val in Hb as

Received: July 8, 2014
Published: October 28, 2014

   dx.doi.org/10.1021/tx5002749 | *Chem. Res. Toxicol.* 2014, 27, 2062−2070

thiohydantoin derivatives.[17,18] With the original modified Edman method, utilizing GC−MS/MS, several background adducts have been disclosed.[1,4] Recently, we have developed a fast LC−MS/MS method, the FIRE procedure, to overcome limitations of the corresponding GC−MS/MS method.[18] The FIRE procedure has been used for detection of known adducts (from acrylamide, glycidamide, and ethylene oxide) in large cohorts using biobank samples.[19,20] Prior to LC−MS/MS analysis, the N-terminal Val adducts are detached as fluorescein thiohydantoin (FTH) derivatives using the isothiocyanate reagent fluorescein isothiocyanate (FITC) (Scheme 1). The

Scheme 1. General Scheme of Detachment and Derivatization of N-Terminal Hb Adducts with Edman Reagent[a]



[a]R_1 represents a covalently bound adduct; R_2 indicates fluorescein, which is the Edman reagent used in the applied FIRE procedure. Hb, hemoglobin.

detachment with modified Edman degradation procedures is considered to be specific because the detachment of non-adducted Val occurs to a much lower extent.[21,22] Multiple-reaction monitoring (MRM) is used in the MS/MS analysis to achieve as good level of detectability as possible of adducts at low levels. With the FIRE procedure, one previously unknown adduct present in human Hb has been identified, namely, an adduct from methyl vinyl ketone.[23]

In the present study, we extend the FIRE procedure to enable the screening of *a priori* unknown adducts in human Hb. From previous work with the FIRE procedure, it was clear that all N-terminal Val adducts studied as FTH derivatives exhibited similar fragmentation pathways in the LC−MS/MS analysis, resulting in at least three common fragments.[18,23] This implies that we expect any adduct to exhibit the same fragmentation pathways. On the basis of this, we set the screening methods to screen human blood samples within a specific mass range, covering a span of 135 Da. The chosen mass range covers any modification ranging from methylation (a mass increment of 14 Da) up to an added mass of 149 Da, thereby including all N-terminal Hb adducts previously studied using modified Edman degradation procedures. Four fragmentation pathways, common for the FTH derivative of N-terminal Val adducts, were utilized. In this study, 12 human blood samples were analyzed, of which six were from smokers and six were from nonsmokers. The aim of the present work was to investigate the performance of an MS/MS screening method for the detection of unknown Hb adducts isolated with the FIRE procedure and to make a first evaluation of the number of adducts and their levels detected with the method. The ultimate aim is to develop an adductomic approach for the detection of unknown exposures to reactive compounds.

MATERIALS AND METHODS

Caution: *These chemicals are dangerous.* Fluorescein isothiocyanate (FITC), iodomethane, iodoethane, and acrylonitrile are hazardous and should be handled with care.

Chemicals. The analytical standards, fluorescein thiohydantoins (FTHs), formed from N-substituted valine/d_7-valine, were synthesized as described earlier[18,23] and stored in acetonitrile (ACN)/H_2O (1:1, v/v) at −20 °C until use. The complete name of an FTH derivative of Val is fluorescein-5-(4-isopropyl-2-thioxo-imidazolidin-5-one). The following FTH standards, corresponding to adducts from acrylamide (AA), glycidamide (GA), ethylene oxide (EO), and methyl vinyl ketone (MVK), respectively, were used: fluorescein-5-[4-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one] (AA-Val-FTH), fluorescein-5-[4-d_7-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one] (AA-d_7-Val-FTH), fluorescein-5-[4-d_7-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one] (GA-d_7-Val-FTH), fluorescein-5-[4-d_7-isopropyl-3-(2-hydroxyethyl)-2-thioxo-imidazolidin-5-one] (EO-d_7-Val-FTH), and fluorescien-5-[4-d_7-isopropyl-3-(butyl-2-one)-2-thioxo-imidazolidin-5-one] (MVK-d_7-Val-FTH).

Fluorescein-5-isothiocyanate (Isomer I), FITC, reagent grade, was from Karl Industries (Aurora, OH, USA). All other chemicals and solvents were of analytical grade and obtained from Sigma-Aldrich.

Study Population/Blood Samples. Blood samples, from six smokers and six nonsmokers, were collected with approval from the Regional Ethical Review Board in Stockholm (nr 96-312) for the identification of unknown adducts to the N-terminus of Hb. The samples were separated into red blood cells and plasma by centrifugation (10 min, 4500*g*). The red blood cells were washed three times with equal volumes of 0.9% sodium chloride followed by centrifugation and were then lysed by the addition of an equal volume of distilled water before storage in a freezer (−20 °C).

Commercial human blood and plasma used for calibration curves and control experiments were bought from Komponentlaboratoriet, Karolinska University Hospital Huddinge (Stockholm, Sweden). A fraction of the blood was separated into red blood cells and plasma by centrifugation, and the red cells were washed as described above before storage in a freezer (−20 °C). Bovine blood (with citrate) was obtained from Håtunalab (Bro, Sweden).

Sample Preparation. The blood samples were prepared for analysis according to the adduct FIRE procedure for LC−MS/MS analysis[18] (the trademark belongs to Adduct Analys AB, Skolvägen 18, Enebyberg, Sweden). Prior to derivatization with FITC, the Hb content was measured with a HemoCue instrument. The Hb concentration in the 12 screened samples ranged from 104−148 g/L, with an average concentration 121 g/L. In the human and bovine blood samples used as controls and used for the calibration curve, the Hb content was adjusted with water to approximately 140 g/L.

For each individual blood sample, duplicates of 0.25 mL of hemolyzed red blood cells were derivatized with FITC (5 mg) at 37 °C during mixing (with 750 rpm) overnight. Deuterium-substituted internal standards (IS), AA-d_7-Val-FTH, GA-d_7-Val-FTH, EO-d_7-Val-FTH, and MVK-d_7-Val-FTH, were mixed into the blood samples (5 pmol/sample). The proteins were precipitated with ACN (1.4 mL), after which the samples were centrifuged (10 min at 11 000 rpm). The samples were then alkalized with 0.5 M ammonium hydroxide, transferred to mixed-mode anion-exchange SPE columns, washed with ACN, H_2O, and 0.5% cyanoacetic acid in H_2O, and then eluted with 0.25% cyanoacetic acid in ACN (1.4 mL). The samples were evaporated to dryness using air and then redissolved in H_2O/ACN (200 μL, 6:4, v/v) prior to LC−MS/MS analysis.

Equipment for Sample Preparation. The SPE cartridges, Oasis Max (3 cc, 60 mg, 60 μm), were obtained from Waters (Milford, MA, USA). A Thermomixer comfort and a 5804 R centrifuge with rotor F-45-30-11 (Eppendorf Nordic, Denmark) were used for the workup of blood samples derivatized with FITC. The Hb analyzer (Hb 201+) was obtained from HemoCue (Ängelholm, Sweden).

LC−MS/MS Instrument and Conditions for Analysis. The LC−MS/MS system consisted of a Shimadzu Prominence LC 20 system (Shimadzu Corp., Kyoto, Japan) interfaced to an API 3200 Q-trap instrument with a turbo TurboIonSpray interface (electrospray ionization) from AB Sciex (Concord, ON, Canada). A Discovery HS C18 column (3.0 μm, 2.1 mm × 150 mm), with a Discovery HS C18 guard column (3.0 μm, 2.1 mm × 20 mm) (Supelco Analytical, Bellefonte, PA, USA), was used for LC separation. The mobile phase consisted of A (0.1% formic acid in H_2O/ACN (95:5, v/v)) and B (0.1% formic acid in H_2O/ACN (5:95, v/v)). A gradient was applied,

dx.doi.org/10.1021/tx5002749 | Chem. Res. Toxicol. 2014, 27, 2062−2070

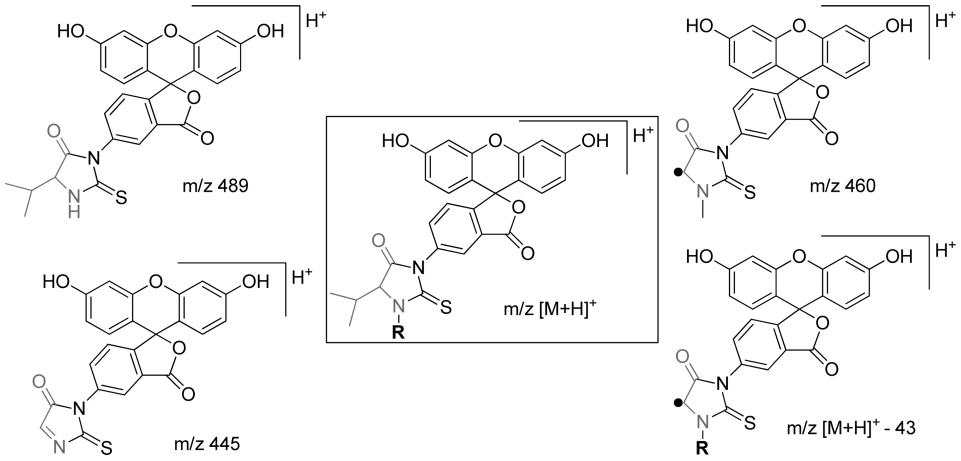


Figure 1. General structure of an FTH derivative of a valine adduct is depicted in the center. In the corners, the four common fragments that were used for screening are shown. The gray-colored parts represent the detached N-terminal Val.

starting from 20% B and increasing to 100% B in 25 min. The final composition was kept for 5 min before re-equilibrating the column for 5 min prior to the following injection. The injection volume was 15 μL, and the flow rate was 120 μL/min. The MS instrument was used with the following settings: declustering potential, 95 V; entrance potential, 6 V;, collision energy, 50 V; ion source gas 1 (N_2), 30 arbitrary units (au); ion source gas 2 (N_2), 20 au; curtain gas (N_2), 20 au; collision gas (N_2), 5 au; ion spray voltage, 5000 V; and vaporizing temperature, 450 °C. Data acquisition was performed in positive ion and profile scan mode using multiple-reaction monitoring (MRM) at unit resolution (0.6−0.8 Da full width at half-maximum). The same MS/MS parameters were used as those for previously identified adducts, with minor adjustments.[18,23]

The studied mass range included 135 mass units, from m/z 503 to m/z 638, with the lowest m/z corresponding to a methylated valine. To cover the mass range and include four possible fragments (m/z 445, m/z 460, m/z 489, and $[M + H]^+ - 43$) for the potential FTH analytes (Figure 1), each sample was injected 12 times. Each of these 12 analyses covered a specific part of the precursor ion mass range (Figure 2), and the instrumental settings were kept the same. Each

Q1 → Q2 → Q3

Q1: FTH analyte mass	Q3: Fragments			
[M+H]+	445	460	489	[M+H]+ - 43
503	445	460	489	460
504	445	460	489	461
505	445	460	489	462
506	445	460	489	463
.	.	.	.	.
.	.	.	.	.
638	445	460	489	595

Figure 2. Mass range studied covered 135 mass units, from m/z 503 to m/z 638. For each precursor ion, four MRM transitions were included in the MS/MS methods.

analysis included 50 MRM transitions, of which 46 were unique for each method. In each MRM method, transitions for AA-Val-FTH, AA-d_7-Val-FTH, GA-Val-FTH, and GA-d_7-Val-FTH were included. Transitions for EO-Val-FTH, EO-d_7-Val-FTH, MVK-Val-FTH, and MVK-d_7-Val-FTH were included once for each sample. The dwell time for each MRM transition was 20 ms.

Quantification/Calibration Curve. For each sample, the 12 separate chromatograms were individually evaluated, and all fragment peaks exceeding 100 cps were manually integrated. All peaks observed were initially considered as corresponding to possible adducts. The retention time and the area of the peak were used for evaluation. The average peak area of the four possible transitions was used for quantitative evaluation of each adduct candidate. For each sample, the peak areas were adjusted for the measured Hb concentration. To transform these values to a more useable format, a semiquantitative approach was applied. Using a simplified assumption that the FTH analyte of all N-substituted valines gives a similar response in the MS analysis, a calibration curve using peak area ratios of AA-Val-FTH/AA-d_7-Val-FTH was used to quantify assigned Hb adducts. The standards were prepared by adding AA-Val-FTH diluted in ACN/H_2O 6:4 as follows: 0.15, 0.31, 0.63, 1.25, 2.5, 5.0, 10.0, and 20.0 pmol to 250 μL of bovine blood. The samples of bovine blood were spiked with the standards and the IS immediately after derivatization.

Study of Fragmentation Patterns. All precursor ions that yielded two or more of the four specific fragments in the 12 analyzed blood samples from smokers and nonsmokers were further investigated by LC−MS/MS, monitoring in product ion scan mode (using the ion trap detector of the same instrument and collision energy of 60 V; all other settings and chromatography were the same as those given above).

Control Experiments. Control experiments, using a targeted MRM method based on the findings of the screening experiments, were performed to check for possible *in vitro* formed adducts or interfering compounds, obtained in the workup including FITC derivatization. Duplicate control experiments including workup were performed on equine myoglobin and human serum albumin (HSA) (37 mg in 250 μL and 150 mg in 1.5 mL, respectively; plasma was used as solvent to facilitate dissolution). The molar concentrations of these proteins in the control samples were approximately the same as that for Hb in the analyzed blood samples. Human blood plasma without added protein, that is, with lower protein concentration, was used as a further control. In addition to the above-mentioned controls, three individual human blood samples and three bovine blood samples were analyzed as reference samples. This was done to confirm that adduct candidates were also present in fresh blood samples, not just in blood that had been freeze-stored for years, and to investigate if detected adduct candidates were also observed in bovine blood or were related only to human blood. The two most intense fragments of each adduct candidate as well as of the known adducts were monitored, and transitions for the internal standards were included. To cover all adduct candidates, each sample was injected twice.

Preparation of FTH Analytes of Known Adducts from *in Vitro* Reactions with Blood. One mL portions of human red blood cell lysate (nonsmoker, 137 g Hb/L) were incubated with acrylonitrile in water and iodomethane or iodoethane in dichloromethane. Incubations were done with the individual compounds at concentrations of 250 μM and 500 μM. The samples were incubated for 3 h at 37 °C with mixing at 750 rpm. Portions of 250 μL of the incubated samples were then derivatized and treated as the samples described in

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Scheme 2. Overview of the Strategy for Screening of Unknown Adducts Used for the Study

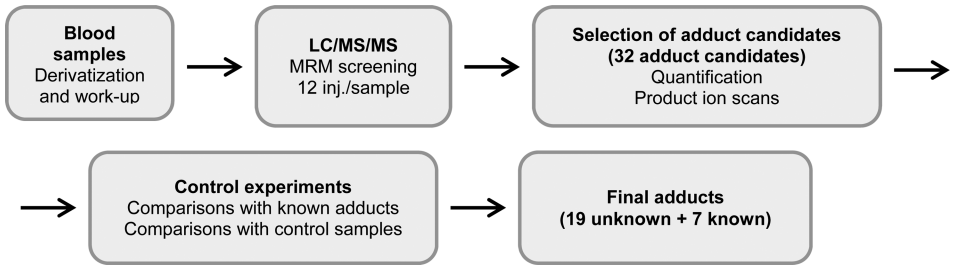

Table 1. Relative Peak Intensities (%) of Diagnostic Ions from Product Ion Scans of 7 Known and 14 Unknown Adducts

				relative intensities (%)				
precursor ion (m/z)	t_R (min)	added mass from modification (Da)[a]	most abundant ion (m/z) (100%)	precursor ion	445	460	489	precursor ion −43
Known Modifications								
503,[b] Methyl	18.3	14	390	7	18	34	n.d.[d]	34
517,[b] Ethyl	19.5	28	390	56	50	12	n.d.	34
533,[b] Ethylene oxide	15.9	44	390	35	29	17	6	15
542,[b] Acrylonitrile	18.0	53	390	56	41	5[c]	7	22
559,[b] Methyl vinyl ketone	18.4	70	390	26	42	2[c]	16	17
560,[b] Acrylamide	14.7	71	390	34	49	n.d.	19	30
576,[b] Glycidamide	14.0	87	576	100	12	26	23	35
Unknown Modifications								
520	20.0	31	434	44	n.d.	22	n.d.	12
547	15.9	58	390	20	57	30	1[c]	11
561	16.2	72	390	42	54	n.d.	15	30
561	23.0	72	390	17	11	13	n.d.	
573	19.8	84	390	42	40	3[c]	25	16
575	24.4	86	390	23	14	18	n.d.	n.d.
577	14.7	88	390	50	25	36	18	8
580	22.4	91	580	100	46	n.d.	12	15
593	13.2	104	390	74	13	17	17	n.d.
595	18.8	106	445	47	100	n.d.	38	n.d.
615	23.8	126	615	100	21	n.d.	40	5[c]
617	16.5	128	617	100	27	18	46	8
625	15.8	136	625	100	25	65	10	n.d.
631	16.9	142	631	100	15	n.d.	55	13

[a]Added mass of modification is calculated by subtracting 489 (m/z of fragment corresponding to FTH derivative of nonmodified Val) from the m/z of the $[M + H]^+$. [b]$[M + H]^+$. [c]For ions with relative intensity, 1−5% the S/N is 5−11. [d]n.d. = not detected.

the Sample Preparation section. The derivatized samples were subsequently analyzed by LC−MS/MS utilizing the four MRM-transitions expected for the formed adducts. Product ion scanning was also performed to obtain the fragmentation patterns (using the ion trap detector as described in the Study of Fragmentation Patterns section).

RESULTS

Overview of the Screening Strategy. The design of the study is schematically given in Scheme 2. Blood samples from six nonsmokers and six smokers were derivatized according the FIRE procedure to detach adducts with N-terminal valines in Hb. These samples were then screened for adducts on the basis of specific diagnostic fragments by LC−MS/MS. Each sample was injected repeatedly to cover the entire mass range (m/z 503−638) studied. Peaks assigned as possible adduct candidates were further examined by LC−MS/MS in the product ion scan mode. The fragmentation pathways were compared with known adducts. Control experiments were also performed to check for artifacts, such as interfering compounds originating from sample processing.

The mass range of 135 Da was primarily chosen because it includes all Hb adducts previously studied using modified Edman procedures. Before the final selection of the mass range, a smaller number of samples had been screened for an additional 45 Da (m/z 639−683), without detecting any likely adduct. Thus, to have a reasonable limit for this first evaluation of the approach, we used the 135 Da mass range.

Selection of Adduct Candidates. Every sample generated 12 different mass chromatograms. Those were evaluated individually, and all peaks of intensities above 100 cps were integrated manually. Background noise observed in blank injections was approximately 60 cps throughout the whole gradient. The first selection of adduct candidates was based on the criterion that at least two specific fragments (of the four diagnostic fragments monitored) should be present at the same retention time. The screening of the 135 studied precursor ions resulted in 26 unidentified analytes that fulfilled this criterion in addition to peaks corresponding to six previously known adduct

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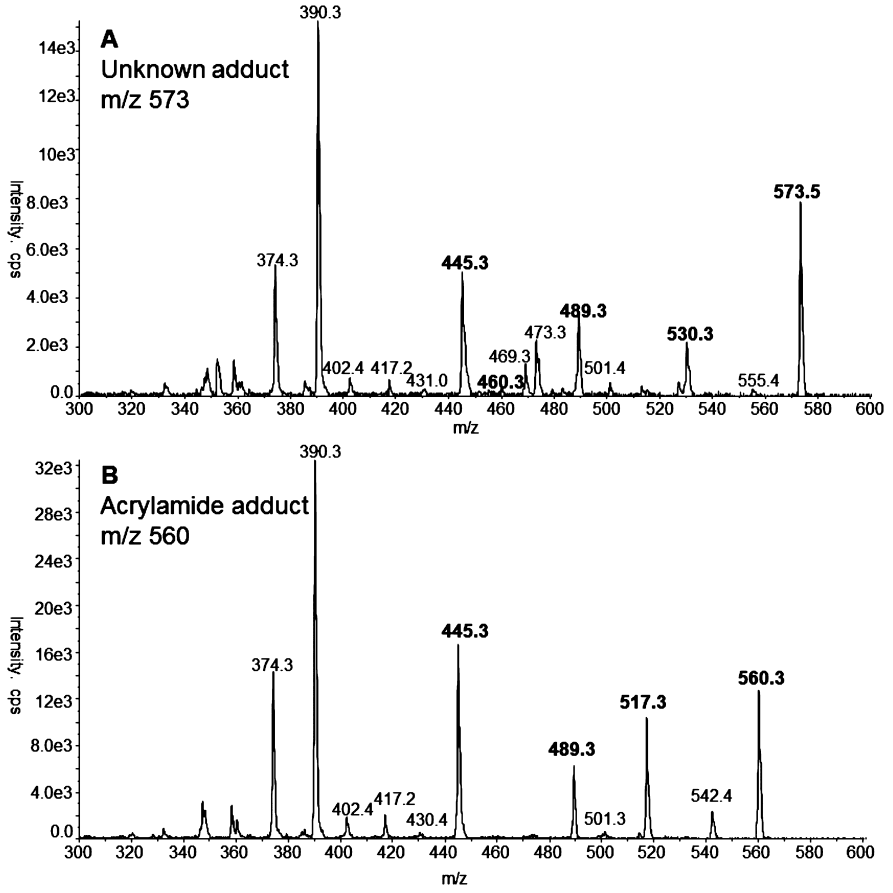

Figure 3. Product ion spectra for (A) unknown adduct m/z 573 and (B) AA-Val-FTH (m/z 560). Precursor ions and fragments used in MRM methods are given in bold. Fragment ions m/z 374 and m/z 390 have low diagnostic value (they contain no or low adduct-specific information) and were therefore not used for screening, even though the intensities are relatively high.[18]

analytes. This means that a total of 32 possible adducts were observed within the studied mass range. From the screening data and *in vitro* incubation experiments, an analyte corresponding to the ethyl adduct (m/z 517) could be confirmed and added to the known adducts, making the total number of known adducts observed seven. From fragmentation patterns obtained in the product ion scan mode and the results from the control experiments, six of the adduct candidates were considered to be interfering compounds. Although their fragmentation patterns exhibited some of the diagnostic fragments, the general patterns were not in concordance with what was expected from known adducts, and these adduct candidates were also observed in the control experiments with myoglobin and HSA. These six compounds are hereafter excluded from the final selection of adduct candidates. The experiments using bovine blood as reference samples exhibited methyl, ethyl, and ethylene oxide adducts and three of the final unknown adducts. Overall, the levels in bovine blood were much lower than those in human blood. Altogether, 26 adducts were detected: 19 unknown and 7 identified adducts. Details of the results of the control experiments are given in the Supporting Information.

Fragmentation Patterns of Known Adducts and Unknown Adducts in Product Ion Scan Mode. The fragmentation patterns in product ion scan mode were investigated for the 19 unknown adducts as well as for the previously known adducts. Of the unknown adducts, 14 yielded interpretable fragmentation patterns in product ion scan mode. The relative peak intensities for the four fragments monitored in the MRM methods are presented in Table 1. As an example, Figure 3 displays the product ion spectrum for one of the unknown adducts (m/z 573) and for the adduct from acrylamide (m/z 560).

Levels of *a Priori* Known Adducts in Human Blood Samples. Standards of FTH-Val derivatives of acrylonitrile, ethyl, and methyl adducts were prepared as described in the Materials and Methods section by the incubation of blood with the corresponding electrophiles. The adducts were identified in the samples based on the retention times and fragmentation patterns. For adducts from acrylamide, glycidamide, ethylene oxide, and methyl vinyl ketone, corresponding deuterium-substituted ISs were used for the identification. The adduct levels, expressed as picomoles per gram of Hb, of both the unknown and known adducts, except for the acrylamide adduct, were estimated by using a semiquantitative approach. This implied that the average response for the different monitored fragments was used and quantification was performed using the calibration curve for AA-Val-FTH/AA-d_7-Val-FTH. This was considered to be reasonable, as all known Val-FTH adducts so far studied have been found to exhibit a similar response factor under the applied LC−MS/MS conditions. The precursor $[M + H]^+$ ions corresponding to FTH derivatives of known Hb adducts were m/z 503 (methyl), m/z 517 (ethyl), m/z 533 (from ethylene oxide), m/z 542 (from acrylonitrile), m/z 559 (from methyl vinyl ketone), m/z 560 (from acrylamide), and m/z 576 (from glycidamide). The estimated average levels of

dx.doi.org/10.1021/tx5002749 | *Chem. Res. Toxicol.* 2014, 27, 2062−2070

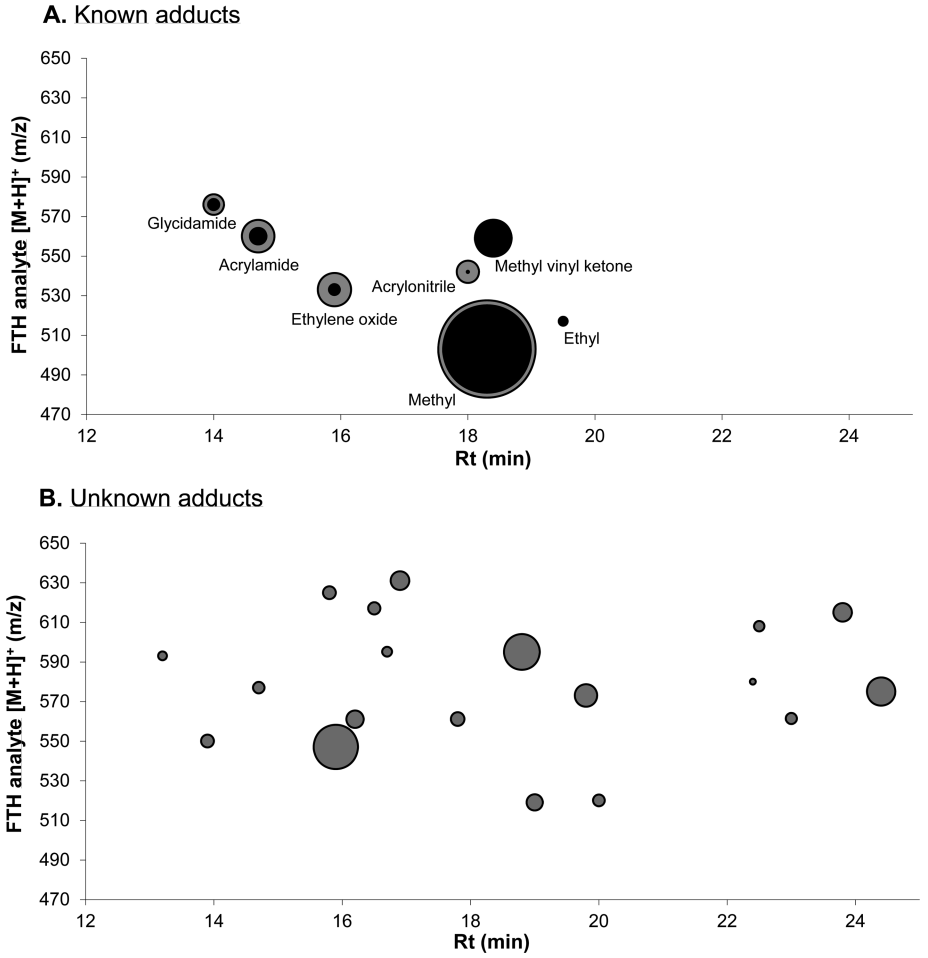

Figure 4. Adductome maps showing the relative average levels of (A) seven previously identified adducts detected in smokers (n = 6, gray) and nonsmokers (n = 6, black) and (B) the 19 unknown adducts detected in smokers and nonsmokers (n = 12). The retention times (min) are shown on the x axis, and m/z of the FTH analyte precursor ion, on the y axis.

these adducts observed in Hb from smokers and nonsmokers are displayed in Figure 4A in an adductome map format. The levels ranged from about 10 pmol/g Hb (the ethyl adduct) to 1200 pmol/g Hb (the methyl adduct). The estimated average level of AA-Val-FTH was 35 pmol/g Hb for nonsmokers (n = 6) and 135 pmol/g Hb for smokers (n = 6). The corresponding levels of GA-Val-FTH were 15 and 54; of AN-Val-FTH, 1 and 62; and of EO-Val-FTH, 15 and 140 pmol/g Hb in nonsmokers and smokers, respectively. The other known adducts showed no significant difference between the groups, and the levels were as follows: for Ethyl-Val-FTH, 10 and 10; for Methyl-Val-FTH, 970 and 1200; and for MVK-Val-FTH, 171 and 156 pmol/g Hb in nonsmokers and smokers, respectively.

Estimated Levels of Unknown Adducts in Human Blood Samples. Considering both the fragmentation patterns and the results from the control experiments, 19 compounds observed in the human blood samples remained as strong candidates of unknown adducts. Their relative levels (average for all 12 subjects) are shown in an adductome map format in Figure 4B. The concentration range of the unknown adducts was estimated to be 5−250 pmol/g Hb and showed no significant difference between smokers and nonsmokers.

Statistical Considerations. Student's t-tests were performed to investigate whether the concentration of any unknown adduct differed significantly between smokers and nonsmokers. Significantly higher levels of acrylamide, glycidamide, ethylene oxide, and acrylonitrile adducts were observed in smokers, whereas no significant difference were observed in the levels of methyl, ethyl, and methyl vinyl ketone adducts (5% significance level, two-sided t-test). However, for most of the unknown adducts, the levels were similar for both sample groups. For only two of the analytes, m/z 547 and 595, were the concentrations significantly different at the 5% significance level (m/z 547 was higher in nonsmokers and m/z 595 was higher in smokers). Because of the small differences between individual samples regarding the levels of unknown adducts, we chose to present the mean values of both smokers and nonsmokers in Figure 4B.

DISCUSSION

The presented LC−MS/MS screening method was shown to be promising for the detection of unknown adducts, as 19 likely Hb adducts to N-terminal valine were ultimately indicated. None of those had been previously detected in human blood with this method. For some of them, the levels were relatively high and comparable with levels of known background adducts.[19,23] The estimated concentrations were all within the range 5−250 pmol/g Hb, with 17 analytes being between 10 and 100 pmol/g Hb. No difference in the levels of unknown adducts was observed between smokers and nonsmokers, and from the results, it cannot be concluded whether any of the unknown adducts are related to smoking. However, it is

dx.doi.org/10.1021/tx5002749 | Chem. Res. Toxicol. 2014, 27, 2062−2070

plausible that the observed unknown adducts reflect other exogenous or endogenous sources of background exposure. To reveal small differences between smokers and nonsmokers in the observed levels of Hb adducts, a much larger set of samples would be needed.

The highest concentration measured in this study was found for the methyl adduct, to which, according to our earlier studies, *S*-adenosylmethionine is the predominant background source.[24] A slightly higher level of this adduct, probably due to other methylators, has been previously found in smokers (in a study of twins discordant for smoking).[25] Our earlier work also suggests that the background level of the *N*-(2-hydroxyethyl)-valine adduct mainly originates from endogenous ethene, with intestinal flora and lipid peroxidation as important determinants[4] and with increased levels of the adduct in smokers due to ethene in tobacco smoke.[5] Intake of food cooked at high temperatures is the source of adducts from acrylamide and its metabolite glycidamide.[6,26] Acrylonitrile adducts are barely detected in nonsmokers but show increased levels in smokers due to acrylonitrile from tobacco smoking.[7,27] An adduct from methyl vinyl ketone has been identified earlier and is regularly observed in human blood samples.[23] It has not yet been proven if the source is endogenous, exogenous, or due to formation at workup or if the presence is due to a combination of these sources. To our knowledge, background levels of ethyl adducts in human Hb have not been previously reported; however, there are several reports of ethyl adducts in DNA that demonstrate a connection between adduct levels and alcohol consumption.[28−31] The ethyl adduct in DNA (N^2-ethyl-dG) is thought to form from the *in vivo* reduction of the ethylidene adduct (N^2-ethylidene-dG), the major adduct formed from acetaldehyde during the metabolism of ethanol.[31] In several studies, the ethylidene adduct has been reduced *in vitro* using $NaBH_3CN$ and quantified as the more stable ethyl adduct.[28,30] One source of the observed level of the ethyl adduct in Hb could thus be alcohol consumption.

The observation of three (m/z 547, m/z 561, and m/z 595) of the 19 unknown adducts in bovine blood, although at very low levels, confirms that some of the unknown adducts are not unique to humans. The adduct corresponding to ethylene oxide as well as the methyl and ethyl adducts are regularly observed in bovine blood analyzed with the FIRE procedure (data not shown).

The control experiments could not be taken as a complete test to exclude false adducts. Adducts formed *in vitro* could still be falsely assigned as *in vivo* formed adducts. *In vitro* formation of adducts is a difficulty known to be associated with some adducts and could be a problem with any method of measurement. For instance, from our earlier work, it is known that some adducts could be formed during the storage of blood, as shown for the adduct from ethylene oxide.[32] A method tested here was also to utilize a valine-derivative/peptide to monitor possible artificial formation of adducts during the analytical procedure (data not shown). However, it was found that none of the commercial valine-derivatives/peptides used had the extreme purity required. The detected adducts in such experiments are most likely due to the very high efficiency of the FIRE procedure to detect N-substituted valines, making it very sensitive to contaminations of N-substituted valines when synthesized valine-derivatives are undergoing the FIRE procedure. In Hb, the detection of an adduct level of 10 pmol/g Hb corresponds to 0.16 modified N-terminal valines per 10^6 Hb molecules. This would correspond to the detection of about 1 N-substituted valine among 10^7 unsubstituted valines, which means that an extreme purity of valine is required to make it useful as a control in the derivatization with FITC.

The presented approach is only one of several strategies to be explored for adductomics. Different approaches will have different advantages and limitations. One major advantage with N-terminal valine as the nucleophilic target site is that adducted N-termini cannot be misincorporated during protein synthesis, that is, misincorporation is not a route to *in vivo* formed adducts.[33] Furthermore, the FIRE procedure makes it possible to determine hydrophilic, thermolabile, and nonvolatile adducts of higher molecular weight, which was not possible with the earlier N-alkyl Edman GC−MS/MS method.[17,21] On the other hand, the latter method has so far shown a higher detectability of adducts and is also able to measure background adducts, such as those corresponding to propylene oxide[4] and glycidol.[34,35] No method based on a modified Edman degradation procedure can be applied when the valine N is blocked for the reaction with the reagent, as in the case of the ring-closed adduct from diepoxybutane.[36−38] Adducts that are unstable *in vivo* constitute another difficulty. Examples are Schiff bases, which have to be stabilized by reduction *in vitro* prior to analysis.[39]

The number of detected unknown adducts in this study is sufficiently small that they can be handled with in-depth studies to verify the identity and to investigate the source of each unknown adduct. According to our experience, final proof of a true *in vivo* formed adduct requires verification of its identity and a quantitative relationship to endogenous or exogenous exposure sources.[6,40] Future work will focus on the identification of the observed adducts. Our hypothesis so far is that they primarily originate from compounds of relatively high reactivity toward nitrogen and sulfur atoms, for example, those leading to Michael addition products. On the basis of the hypothesis about the identity of an adduct-forming electrophile, reference compounds will be generated in different ways. Other possibilities could be increasing the concentration of selected unknown adducts for the collection of more detailed fragmentation patterns. High-resolution MS could also be useful, as knowledge of the elemental composition of the unknown adducts would be of considerable help for structure deduction. Balbo et al. recently used high-resolution MS in combination with MS/MS/MS for DNA adductomics,[41] which shows the power of advanced MS technologies for improved selection of adduct candidates. Another further development is to improve the detectability of adducts with the used analytical methodology, the FIRE procedure.

Several papers on adductomics concern method development using samples treated with adduct-forming compounds or with added standards.[41−44] A few studies of background adducts in human samples are published. In all adductomic studies, the identification of observed putative adducts is a challenge, and the identification of background adducts is more difficult than the identification of adducts formed in treated samples. Matsuda and co-workers, who have published several pioneering works on DNA adductomics, were able to detect seven background DNA adducts in nontreated human lung and esophagus.[45] The identification was done by comparisons with authentic standards and stable isotope dilutions. Among the observed adducts was ethylated deoxyguanosine (N^2-ethyl-dG), which most probably is related to the Hb ethyl adduct observed in the present study. The other identified adducts were

oxidative modifications; however, all of them had been previously observed. The same approach was later used by the same group to detect seven lipid peroxidation-related DNA adducts in human gastric mucosa samples.[46] Rappaport and co-workers improved their original screening method for Cys34 adduct in HSA[47] by the use of high-resolution MS and have been able to identify eight peptide modifications based on accurate masses in samples from nonsmokers.[16] The modifications included oxidation products at Cys34, loss of water and Lys, cysteinylation, and transpeptidation of Arg. From these early results, it seems that different nucleophilic targets could be used to study different classes of adducts, meaning that several methods could be used in parallel to cover a larger range of exposures.

The presented screening approach is meant to be one method of several in the toolbox for characterization of the load of exposure to electrophilic compounds in humans and for investigation of the possible importance of these compounds as contributors to chronic diseases, e.g., by comparing the levels in different populations regarding age, health, life-style, and exposure. Even if most adducts are so far unidentified, adductome maps might reveal significant differences that are interesting *per se*. Identification of adducts, on the other hand, may lead to discovery of previously unknown internal or external exposure sources. Disclosure of exposure sources is expected to be a challenge, and different strategies have to be considered.

■ ASSOCIATED CONTENT

Supporting Information

Full details of control experiments and summary of the data on preliminary adduct candidates observed in human blood that were also observed in control experiments with bovine blood, myoglobin, or HSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

The research was funded by the Swedish Research Council (VR), the Swedish Cancer and Allergy Foundation, and Stockholm University.

Notes

The authors declare the following competing financial interest(s): H.v.S. and M.T. are stakeholders in Adduct Analys AB, Stockholm, Sweden, the company that own the patent for the analytical method used to screen hemoglobin adducts.

■ ACKNOWLEDGMENTS

Dr. Per Rydberg is acknowledged for invaluable contributions during the development of the FIRE procedure.

■ ABBREVIATIONS

AA, acrylamide; ACN, acetonitrile; AN, acrylonitrile; EO, ethylene oxide; FITC, fluorescein isothiocyanate; FTH, fluorescein thiohydantoin; GA, glycidamide; Hb, hemoglobin; HSA, human serum albumin; IS, internal standard; MRM, multiple reaction monitoring; MVK, methyl vinyl ketone; t_R, retention time; SA, serum albumin; SPE, solid-phase extraction

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