

Announcement by the German Federal Environmental Agency

Use of hemoglobin adducts as biomarkers to monitor exposure to genotoxic substances

Opinion of the Human Biomonitoring Commission of the German Federal Environmental Agency

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Introduction

The role of biochemical effect monitoring as part of human biomonitoring has already been described elsewhere [1]. That contribution also discussed the reasons. There it has been also discussed why protein adducts may be used as biomarkers to quantify internal exposure and why the results provide information about the internal exposure and individual kinetics comparable to that obtained from the analysis of DNA adducts. Hemoglobin adducts have already been used as effect biomarkers for quite some time. Currently, hemoglobin adducts from two classes of substances relevant to occupational and environmental medicine are predominantly analysed, hemoglobin adducts of alkylating agents and of aromatic amines including nitroaromatic compounds. Many chemicals of these classes are classified as carcinogens or suspected carcinogens. It is extremely difficult if not impossible to rationalise scientifically limit values or safe exposures to genotoxic carcinogens. It is therefore hard to set human biomonitoring (HBM) values for such substances. This makes it all the more important to control the occurrence of these substances in environmental media, foodstuffs and consumer products, characterise background exposures, quantify the impact of particular exposures on the internal exposure and response of affected persons and assess

the incremental increase in internal exposure and response caused by specific exposures. Examples from the literature are compiled in Table 1. The Human Biomonitoring Commission of the German Federal Environmental Agency recommends that hemoglobin adducts should be used more extensively as biomarkers to control internal exposure and response to genotoxic substances. The rationale for this recommendation is presented below.

Experience with hemoglobin adducts as effect biomarkers

In order to determine the concentration of specific hemoglobin adducts it is necessary to obtain a cleavage product from the protein which can be extracted and separated from the large protein excess. Although reactive molecules may react with several amino acids of the protein, two reaction sites predominate: the free amino group of terminal valine and the SH group of cysteine. The adducted valine can be cleaved off by a modified Edman degradation reaction, which is otherwise used to gradually break down proteins [2]. In the case of cysteine adducts, the bound residue is released hydrolytically [3].

Alkylating agents

Effect monitoring of genotoxic substances began with investigations by Ehrenberg et al. [4] who studied the effects of ethylene oxide, which forms *inter alia* hydroxyethyl adducts with terminal valine. Starting with these investigations the “target-dose concept” has evolved, in which the adduct represents the biologically effective dose at the target structure.

Ethylene oxide is present in cigarette smoke. Accordingly, smokers have higher hemoglobin adduct levels (average of 170 pmol/g of globin) than non-smokers (average of 20 pmol/g of globin). The adduct levels in non-smokers indicate widespread background exposures. Ethylene formed endogenously may contribute to this background.

In addition ethylene oxide is used as disinfectant in sterilizers. Personnel may be exposed to it when opening sterilizers. Correspondingly high adduct levels were occasionally found. Patients treated with cytostatic drugs whose action is based on transfer of hydroxyethyl groups likewise exhibit strongly elevated adduct levels. For instance, adduct levels in the range of 330 pmol/g of globin were determined in patients treated with ACNU (nimustine), a chloroethyl nitroso urea derivative. The highest adduct levels have been found, as expected, in ethylene production workers (up to 16,000 pmol/g of globin).

These examples illustrate the different magnitudes of response. For 2-hydroxyethyl valine, an EKA correlation (EKA = exposure equivalents for occupational exposures to carcinogenic substances) has now been defined, according to which an external ethylene oxide exposure

of 50 ppm (TRK value; TRK = technical guideline concentration) corresponds to an hydroxyethyl valine level of 90 µg/l of blood [5].

The first groundbreaking studies to measure hemoglobin adducts of alkylating agents were performed with histidine adducts. Today, adducts of alkylating agents are determined almost exclusively via the “valine method” [2]. Typical examples with hemoglobin adducts as biomarkers are shown in Table 1.

Alkylating agents studied include: epoxides (ethylene oxide, propylene oxide [6]), unsaturated aliphatic compounds (acrylonitrile [7]), and alkylating agents which are metabolised to epoxides (ethylene, propylene, butadiene, styrene [8]). In addition, methylating substances such as methyl halides and dimethyl sulphate need to be mentioned [9]. At some production sites, valine adducts are now measured routinely as part of exposure control.

Hecht et al. [30] used hemoglobin adducts to demonstrate that tobacco specific nitrosamines are formed in cigarette smoke which are metabolically activated. The reactive metabolites bind to macromolecules which indicates their genotoxic potential.

Arylamines and nitroaromatic compounds

The introduction of effect monitoring for the large group of arylamines, nitroaromatic compounds and azo compounds dates back to observations of Wieland and Neumann [11]. Arylamines and nitroaromatic compounds are metabolically activated to hydroxyl amines, which either as such or after further activation represent the genotoxic metabolites. In erythrocytes, hydroxyl amines are oxidised to nitroso derivatives along with the formation of methemoglobin. The nitroso derivatives preferably react with the SH group of cysteine (93β). Sulfinic acid amides are formed, which are stable in vivo. From these hemoglobin adducts the parent arylamines can be hydrolytically released, and extracted. The cleavage products are identified and quantitatively determined by gas chromatography/mass spectrometry or MS/MS coupled mass spectrometry [12].

For workers in arylamine production plants, amines in urine have been used as biomarkers and blood methemoglobin levels for exposure control for a long time. Both parameters are largely influenced by the sampling time, as levels to be measured change rapidly, within hours or a few days of exposure. This kind of monitoring is important to evaluate the situation in acute poisoning. However, haemoglobin adducts proved to be more suitable to control long-term exposures. The adducts represent the average exposure during the preceding 3 – 4 months.

Table 1
Examples of the use of hemoglobin adducts as biomarkers for humans

Exposure	Adduct / cleavage product	Group studied	Adduct level	Ref.
Alkalating agents				
Ethylene oxide	Hydroxyethyl valine	Smokers (10 cig./day)	120 pmol/ g globin	[34]
		Non-smokers	50 pmol/ g globin	
		Steriliser operators (0.2-8.5 ppm)	16.2 ^a (5.2-32.7) pmol/g globin	[35]
Butadiene	N-(2-hydroxy-3-butenyl) valine	Butadiene production	0.7 (<0.2-1.2) pmol/g globin	[36]
		Controls	0.2 (<0.2-1.5) pmol/g globin	
Acrylonitrile	Cyanoethyl valine	Smokers	88 (75-106) pmol/g globin	[7]
		Non-smokers	< 20 pmol/g globin	
Acrylamide	N-2-Carbamoyl ethyl valine	Industrial workers	63 ^a (11-294) pmol/g globin	[9]
		Controls	28 (16-90) pmol/g globin	
Arylamines				
Aniline (workplace)	Aniline from cysteine adduct	Slow acetylators	123 µg/l blood	[13]
		Fast acetylators	10 µg/l blood	
m-Toluidine	m-Toluidine from cysteine adduct	Non-smokers	187 ng/l blood	[23]
		Smokers, light tobacco	180 ng/l blood	
		Smokers, black tobacco	187 ng/l blood	
4-Aminobiphenyl	4-Aminobiphenyl from cysteine add.	Smokers	154 ± 47 pg/g Hb	[17]
		Non-smokers	28 ± 13 pg/g Hb	
PHIP ^b	PHIP from cysteine adduct	Meat eaters	3.0 ± 0.8 pmol/g Hb	[37]
		Vegetarians	0.3 ± 0.1 pmol/g Hb	
Nitroaromatic compounds				
2,6-Dinitrotoluene	2-Amino-6-nitrotoluene	Former explosives production site	1.5 (0.7-2.1) pmol/g Hb ^c	[28]
		Controls	1.2 (0.7-1.8) pmol/ g H	
2,4,6-Trinitrotoluene	2-Amino-4,6-dinitrotoluene	Former explosives production site	<0.1 (<0.1-8.2) pmol/g Hb ^d	[28]
		Controls	<0.1 (<0.1-1.2) pmol/ g Hb	
1-Nitropyrene	1-Aminopyrene	Garage for buses incl. servicing	0.13 (<0.03-0.58) pmol/g Hb	[19]
		Urban population	0.16 (<0.03-0.68) pmol/g Hb	
		Rural population	0.10 (0.03-0.67) pmol/g Hb	
Sum of 5 polycyclic nitroaromatic compounds ^e		Garage for buses incl. servicing	0.24 pmol/g Hb	[19]
		Urban population	0.26 pmol/g Hb	
		Rural population	0.19 pmol/g Hb	

^a median value and range; ^b PHIP = 2-amino-1-(1-methyl-6-phenylimidazole[4,5-b]pyridine); is formed during preparation of meat and fish (heterocyclic amine); ^c median ± 95% confidence region, difference not significant; ^d median (min.-max.); ^e 1-nitropyrene, 2-nitrofluorene, 3-aminofluoanthrene, 9-aminophenanthrene, 6-aminochrysene

Enzyme polymorphisms may be responsible for individual susceptibility. Slow acetylators have usually higher adduct levels than fast acetylators under comparable exposure conditions as first shown by Lewalter and Korallus [13] (see Table 1).

Hemoglobin adducts have been used to establish a biological tolerance value for occupational exposures (“Biologischer Arbeitsstofftoleranz-Wert”; BAT value) for the first time with aniline and nitrobenzene, the two parent compounds of this class of chemicals (100 µg of released aniline/litre of blood) [14]. This value is considered to correspond with a maximum methemoglobin level of 5% or an aniline concentration in urine of 1 mg/litre (original BAT value). However average occupational exposure and the total burden are better reflected by adduct levels than by the concentration of aniline in urine. Moreover, the BAT value protects also slow acetylators, who – with equal external exposure – react more sensitively than fast acetylators and have, for example, significantly higher methemoglobin levels and thus produce more hemoglobin adducts than the latter.

The analysis of haemoglobin adducts may detect unknown sources as has been demonstrated when the adducts of monocyclic arylamines were analysed in smokers and non-smokers. In this study, relatively high adduct levels were found for m-toluidine, irrespective of tobacco type and smoking status. Numerous other examples of background exposures to specific hemoglobin adducts of various substances are compiled in a report by the Working Group for the Definition of Limit Values in Biological Material of the Deutsche Forschungsgemeinschaft (German Research Association) [15].

It is particularly important to assess the exposure to chemical mixtures. For this purpose it is necessary to identify representative components of the mixture and to measure their adducts. Specific hemoglobin adducts were used to estimate exposure to such complex substance mixtures in various studies (see Table 1) [16]. For example, carcinogenic 4-aminobiphenyl, is present in cigarette smoke; accordingly, smokers were found to have significantly higher adduct levels than non-smokers [17]. As with ethylene dioxide, adducts of this substance have, however, also been measured in non-smokers. This suggests the presence of exposure sources other than tobacco smoke. Hemoglobin adducts of 4-aminobiphenyl may also originate from exposure to 4-nitrobiphenyl, probably present ubiquitously; therefore, they can only be used as effect biomarker in comparison with this background exposure.

Combustion processes in which polycyclic aromatic hydrocarbons (PAHs) are formed usually also generate the corresponding nitroaromatic compounds. Hemoglobin adducts of polycyclic

nitroaromatic compounds, therefore, may be used to estimate exposure to such pyrolysis products, and have been used as biomarkers for coke oven workers [16, 18] as well as for persons exposed to diesel exhaust [19, 20].

Five components were determined individually or given as sum value (see Table 1). For all five nitroaromatic compounds measured, background exposures were so high that the additional exposure of coke oven workers could only be detected in persons working in a particular area of the plant, namely at the bottom of the coke oven. Significantly, among those workers in that highly polluted area with corresponding high exposures with which pollution levels and the corresponding exposures it was most interesting to see that adduct levels in numerous individuals (40% in the case of the 1-nitropyrene adducts) were in the range of variation of the non-exposed control group. It would be interesting to look into the causes of this lower sensitivity. Among the hemoglobin adducts measured, adducts of 1-nitropyrene and 2-nitrofluorene always made up the largest fraction. Both nitroarenes seem to be typical components of pyrolysis mixtures in general. This suggests that hemoglobin adducts of 1-nitropyrene are not a suitable indicator to assess internal exposure and response to diesel exhaust.

Experiences from investigations into internal exposure and response to diesel exhaust in workers at a garage servicing city busses support this assumption. Adducts of all five nitroaromatic compounds were detected, and again, 1-nitropyrene and 2-nitropyrene were predominant. Concentrations were not, however, significantly elevated compared to the comparison groups, as shown in Table 1 [19].

Aromatic amines and nitroaromatic compounds as environmental toxins

El-Bayoumy et al. [21] detected aniline and o-toluidine in the urine of non-smokers and concluded that there must be exposure sources other than tobacco smoke. Ward et al. [22] analysed hemoglobin adducts and concentrations in urine of aniline, o-toluidine and 4-aminobiphenyl from workers at a chemical plant with known bladder cancer excess and came to the same conclusion. In urine analysis, the values of the pre-shift samples differed from those of the post-shift samples, which shows the time-dependence of sampling. Urinary and adduct levels of aniline and, especially, o-toluidine were significantly elevated in workers potentially exposed in the rubber chemicals department although exposure was below occupational limit values. This was not the case for 4-aminobiphenyl, so that, unlike o-toluidine, this substance was ruled out as a causal factor for the bladder tumours at this plant. In addition to the usual differences between smokers and non-smokers, it was again observed, that non-smokers must have been exposed from unknown sources. The same

observation was made by Bryant et al. [23] in their adduct analyses as well as by Grimmer et al. [24], who analysed urine for the carcinogens 2-aminonaphthalene and 4-aminobiphenyl as well as for other amines and found no significant difference between smokers and non-smokers.

Palmiotto et al. [25] determined 9 different aromatic amines in outdoor and indoor air of different buildings and found in a discotheque up to 207 ng/m³ air for the sum of these compounds. Richter et al. [26] determined hemoglobin adducts of 4-aminobiphenyl, o-, m-, p-toluidine and o-anisidine in children from cities with different population. Adduct levels of 4-aminobiphenyl as well as those of some of the monocyclic amines were higher in children from Munich than in children from Augsburg and Eichstätt. Passive smoking increased adduct levels only slightly (not significant).

For residents at contaminated areas at sites of former ammunition production, hemoglobin adducts of dinitro- and trinitrotoluenes (5 cleavage products) were used as biomarkers to detect exposures to explosive-typical carcinogenic nitroaromatic compounds from contaminated soil [27, 28].

In humans, 2,4,6-trinitrotoluene (2,4,6-TNT), the actual explosive, is mainly metabolised to 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT) and 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT). These metabolites also occur in the contaminated soil. Both, TNT and the two metabolites, were detected in the urine of exposed workers who had carried out cleanup operations, whereas none of these substances were detected in the controls [29]. Hemoglobin adducts were found in considerable concentrations in blood samples from workers at a ammunition factory in China. No adducts were found in blood samples obtained from German controls, at a detection limit of 35 pmol/g Hb [30]. In studies which looked into the potential exposure to carcinogenic nitroaromatic compounds of residents in an area comprising land contaminated by explosives, the relevant hemoglobin adducts were well detectable, the detection limit here being lower, at 0.1 pmol/g Hb. For these potential, although presumably lower, exposures [27, 28], values above the detection limit were found for the cleavage products 2-A-4,6-DNT and 4-A-2,6-DNT in, respectively, 40 and 38% of the 47 subjects, but also, surprisingly, in 25 and 21%, respectively, of 48 controls not knowingly exposed (see Table 1). For 2,6-dinitrotoluene, another substance occurring in soil at sites contaminated by explosives, the percentage of samples above the detection limit was over 70% for both the persons potentially exposed and the controls; for 2,4-dinitrotoluene, the corresponding percentage was over 80%. As an example, Table 1 lists 2,6-dinitrotoluene (2,6-DNT), a strong

carcinogen. The results show that background exposure to these nitroaromatic compounds must be widespread.

In the light of these results, it may be questioned whether exposure estimates based on theoretical considerations [31, 32, 33] reflect realistically the exposure situation of residents at this contaminated site. The results of biomonitoring using hemoglobin adducts do not at all, provide evidence that residents of this contaminated site have a higher cancer risk than the controls. They do indicate, however, that there is widespread background exposure to these nitroaromatic compounds, which urgently requires further clarification.

All of these observations support the assumption that arylamines and nitroaromatic compounds are not strictly a workplace problem but instead are much more widespread than was previously assumed, and that they deserve much greater attention when discussing environmental contaminations. For these compounds, as well as for alkylating agents, exposure assessment for any individual substance should be extended to include an assessment of its contribution to total exposure.

Use of the method

For most chemicals which are hazardous to health and which act via reactive metabolites, haemoglobin adducts have been found in the meanwhile. Hemoglobin adduct analysis has matured to the point that appropriately equipped laboratories can now carry it out routinely under the control of quality assurance measures commonly applied today. The use of these parameters in the field of environmental medicine is justified wherever anamnestic or suspicious data from environmental media, foodstuffs or consumer products indicate a possibility that individuals or groups of individuals are exposed. Already, The analysis of hemoglobin adducts has already helped in a number of hazard assessments – e.g. for contaminated areas of former explosives production sites – and provided relevant information that goes far beyond the information obtained by measuring external and internal exposure and, especially, by modelling. Since it becomes increasingly clear that the internal exposure and, more significantly, the internal response cannot be directly inferred from external exposure, the Human Biomonitoring Commission recommends that biochemical effect monitoring should be applied more extensively. Particularly, a broader data base should be created to set reference values for specific hemoglobin adducts. Studies designed to generate such data should exclude, wherever possible, subjects influenced by known exposure sources. Biochemical effect monitoring can help to assess the relationship between total exposure to genotoxic substances and the contribution of individual chemicals or classes of chemicals and to assess the role of chemical mixtures. It may thus serve as a

valuable tool to set priorities for necessary measures to reduce exposure to carcinogenic substances.

Conclusions

The Human Biomonitoring Commission of the German Federal Environmental Agency considers hemoglobin adduct analysis to be a suitable approach to biochemical monitoring of individual exposures to substances forming reactive metabolites and DNA and protein adducts.

The main field of application are exposure assessments for individuals and groups of individuals who are externally exposed to elevated levels of such substances due to special circumstances. Measurement of hemoglobin adducts is a better tool for determining individual exposure and response to the respective substances than, for example, soil analysis or theoretical exposure assessments, which rely on many assumptions.

The Human Biomonitoring Commissions therefore strongly advocates the use of hemoglobin adducts as effect biomarkers whenever there is a need to generate evidence of human exposure to dangerous substances and to assess the exposure of an individual or a group in comparison to a reference population on the basis of reference values (relative risk).

The Human Biomonitoring Commission is of the opinion that the concentration values obtained from adduct analyses do not allow any conclusions to be drawn about the cancer risk (absolute risk). However, where elevated adduct concentrations are measured in exposed persons, this should, from the perspective of environmental hygiene, be taken as signalling the need to take appropriate exposure abatement measures.

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