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Exposure to genotoxic agents: Modified peptides as suitable biomarkers

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Abstract

During the last decades the interest in quantitative assessment of the risks associated with human exposure to industrial toxicants has been growing. Risk assessment for occupational exposure to xenobiotics is carried out by means of environmental and biological monitoring. The latter can be performed by the measurement of biomarkers of the internal dose and biomarkers of effects. The internal dose is measured by the quantification of the intact xenobiotic or some of its main metabolite excreted in biological fluids such as blood or urine. In particular, alkylating agents or their metabolites interact with nucleic acids or other biological macromolecules, like proteins. The quantification of DNA or RNA adducts represents an efficient method for the evaluation of the tissue dose. Adducts can be

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used as effective biological indexes for the biological monitoring of exposure to genotoxic agents.

As there is a precise relationship between the reaction of several chemicals with DNA and macromolecules such as hemoglobin, hemoglobin adducts have been found to be suitable biomarkers for evaluating exposure to toxicants.

Recently a new methodology for the dosimetry of hemoglobin adducts has been proposed. It is based on the quantification of internal modified hemoglobin peptides. Globin chains are digested with specific enzymes and the modified peptides are identified and quantified by Liquid Chromatography / Electrospray Mass Spectrometry. The method was successfully applied in the case of methyl bromide, epichlorohydrin, butadiene and styrene, industrially used toxicants with proved genotoxic effects.

This work reviews the most common biological monitoring procedures, putting in evidence the advantages of using modified peptides as biomarkers of effective biological indexes by illustrating applications and results achieved up to now.

Introduction

The industrialization of more and more countries has been leading to a growing diffusion of exogenous agents both, in general, in the environment and, above all, in workplaces, where highly amount are daily handled. Owing to the potential health risk associated with the absorption of genotoxic agents within the organism, during the last decades the interest in the evaluation of human exposure to these agents has been increasing.

In this review we summarize the most common methods of biomonitoring as well as recent developments in the choice of suitable biomarkers for the biological monitoring of subjects professionally exposed to different categories of genotoxic agents.

Figure 1 reports a schematic view of the metabolic pathway followed by toxicants once they are absorbed from the environment. Xenobiotics are distributed within the organism towards target organs or tissues and undergo to metabolic pathways leading to different biotransformation products. The toxic effect is generally due to the interaction of the intact agent or one of its active metabolites with biological macromolecules such as DNA, RNA or proteins eventually leading to organ toxicity, chromosomal damage and to the initiation of carcinogenic response.

Nowadays the methods available for the biological monitoring of exposure to genotoxic agents are mainly based on two different categories of biomarkers. The first ones allow the evaluation of the *internal dose* absorbed by the organism and consist in the quantification of the xenobiotic itself or one of its metabolites excreted in human biological fluids. The second ones relate with the *effective biological dose* through the determination of the adducts deriving from the interaction of the xenobiotic active form with cellular macromolecules (1-3).

Direct dose monitoring of modified nucleic bases involves a number of analytical problems, principally due to enzymatic repair processes, turnover mechanisms and difficulty concerning the sampling itself. As for many alkylating agents there is a precise relationship between the reaction of several chemicals with DNA and some proteic macromolecules such as hemoglobin (Hb), hemoglobin adducts have been found to be suitable biomarkers for evaluating exposure to toxicants (4-12). Hemoglobin is easy to sample, forms stable adducts which are not subject to repair processes and has a long life time (approx. 120 days in humans) permitting the evaluation of chronic as well as accidental exposure.

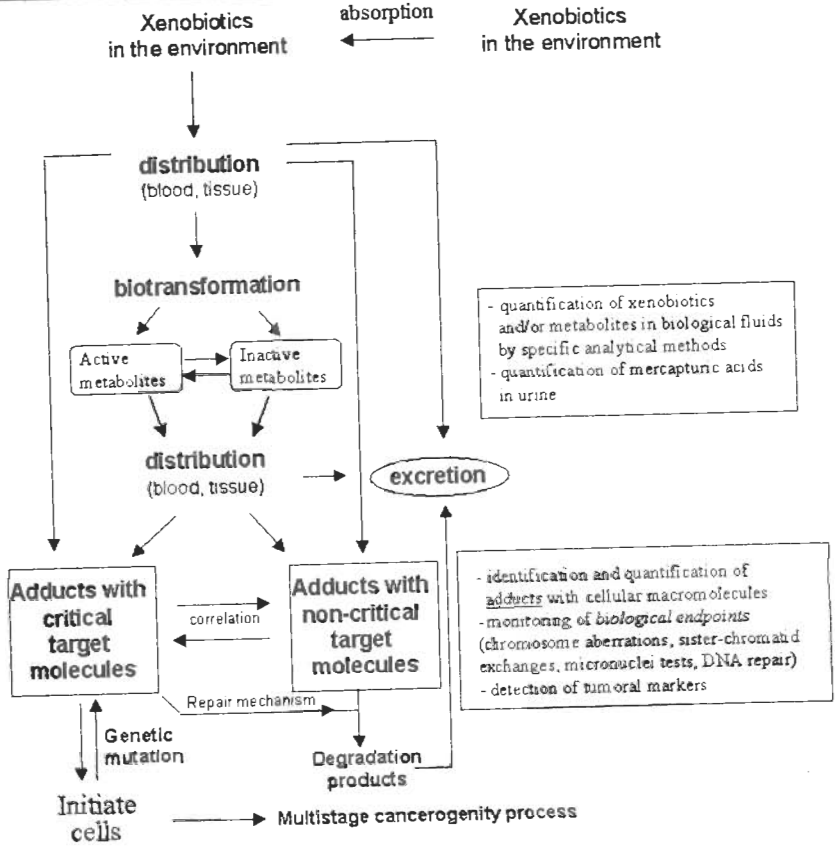

Figure 1. Xenobiotics exposure. Metabolic pathway and biological markers.

used for the biological monitoring of exposed workers (13-15). Methods based on modified Edman degradation allow the dose monitoring of the adducts on the N-terminal Valine of the globin chains. However, depending on the toxicant nature, N-terminal adducts could not be the most abundant ones, because of the presence of eventually more reactive sites within hemoglobin chains. In this case the quantification only of N-terminal adducts would not reveal low exposure levels.

The simultaneous use of biomarkers of different nature (intact xenobiotics, metabolites and macromolecular adducts) and the choice of a specific biomarker allow the accurate evaluation of occupational exposure and, as a consequence, allow to adopt suitable preventive measures, able to avoid health hazard.

Biological monitoring

Toxic agents and/or their metabolites as exposure indexes

According to the half life

could be carried out by extracting from biological fluids unmodified molecules or some of their main metabolites.

As an example, cyclophosphamide, commonly used in the treatment of various kind of cancer is among the most widely used antineoplastic drugs. It is a pro-drug with a half life time of 6-7 hrs and is rapidly transformed to 4-hydroxyphosphamide, aldophosphamide, carboxyphosphamide up to acrolein and mustard phosphoramide, that are the alkylating agents able to cross-link DNA molecules causing therapeutic and also carcinogenic effects (16). Even if the concentration of unmodified cyclophosphamide excreted in urine of exposed workers constitutes only a small fraction of the absorbed dose (17), the parent drug itself has been used in several studies as a suitable marker in estimating the total uptake of the drug. Among the specific methods for the detection of unmodified cyclophosphamide levels in 24 hrs urine, the most suitable have been based on gas or liquid chromatography coupled with nitrogen-phosphorous, electron-capture or mass-selective detection (18-22). Some methods require the preparation of volatile derivatives for gas chromatographic analyses, while HPLC separation techniques avoid derivatization reactions.

As other examples of genotoxic agents widely diffused in chemical industries, organic solvents (benzene or in general aromatic compounds) and highly reactive alkylating agents (for instance epoxides or styrene) have to be mentioned. For organic solvents the high volatility of the substances under investigation can be used for extraction procedures from biological fluids by Head-Space techniques, followed by Gas Chromatographic / Mass Spectrometry (HS-GC/MS) detection methods. In this way unmodified solvents are determined in biological samples, quantified and used as exposure markers (23-28). On the other hand, taken into account the complex metabolism of each compound, even for the biological monitoring of subjects exposed to volatile substances, some metabolites are often considered as specific markers. *t,t*-muconic acid and other benzene metabolites such as hydroquinone and chatecol have been explored and are still used as markers of benzene exposure. As well as the determination of urinary ippuric acid and cresols relate to toluene exposure (29-31).

The interaction between many alkylating agents and glutathione represents an important detoxification pathway (32-34) and involves the formation of specific mercapturic acids deriving from conjugation processes of exogenous agents (or metabolites) with glutathione itself. That is why, mercapturic acids can be used as specific exposure indexes. This is the case of S-phenyl mercapturic acid for benzene exposure (35-37), S-benzyl mercapturic acid for toluene (38,39), N-acetyl-S-(2-phenyl-Hydroxyethyl)Cysteine and 1,2-dihydroxybutyl mercapturic acid, respectively for styrene (40-42) and butadiene (43,44), N-acetyl-S-(2-cyanoethyl) Cysteine mercapturic acid for acrylonitrile (45,46).

Table 1 summarizes the main analytical techniques adopted for the quantification of intact molecules or their metabolites in the biological monitoring of some alkylating agents commonly used in industry or in antineoplastic therapy.

Macromolecular adducts: DNA and hemoglobin

Together with the quantification of unmodified toxic agents or some metabolites, the determination of adducts formed following the interaction with biological macromolecules could give a better insight in the actual exposure of occupationally exposed subjects, giving more information for the risk assessment.

Most chemical exogenous agents (or their metabolites) are electrophilic species

giving rise to 7-substituted guanines as main adducts. Numerous literature data are available showing the DNA-adduct formation following the interaction with different xenobiotics. Phosphoramide mustard, a cyclophosphamide metabolite, is a bifunctional alkylating agents able to cross-link DNA strands between two N-7 guanines (47,48); mono-substitutes epoxides modify exocyclic sites in DNA bases (49-51); different DNA reaction products have been observed from the *in vitro* and *in vivo* exposure with ethylene oxide, N-(2-hydroxyethyl)-N-nitrosourea, vinyl chloride, epichloro-hydrin, butadiene, methyl bromide (52-60).

The *effective biological dose* of electrophilic compounds can be determined from the measurement of the DNA-adducts and several analytical methods have been developed. They could be mainly performed by DNA hydrolysis or ^{32}P-post labeling techniques (61-69). Besides, cytogenetic effects can also be monitored by surveillance of chromosomal damage in human somatic cells: structural chromosome aberrations (CA), sister-chromatid exchanges (SCE), micronuclei (MN) or hypoxanthine-guenine phosphoribosyl transferase gene mutations (HPRT mutation) (70-79).

Because amino acid residues on proteins possess nucleophilic sites of varying strength, it has been demonstrated since 1976 that hemoglobin in peripheral erythrocytes can be adopted as a suitable monitor molecule for dosimetry of electrophilic reagents (4). The

Table 1. Toxic agents (or metabolites) dosimetry

Alkylating agents	Quantified molecule	Analytical technique*	References
Cyclophosphamide	Cyclophosphamide	LC(GC)/MS	18-21
	Cyclophosphamide	GC/NPD	22
Benzene	Benzene	HS-SPME-GC/MS	23,25,27,30
	Metabolites	LC(GC)/MS	29,30,35-37
Toluene	Toluene	HS-SPME-GC/MS	26-28,30
	Metabolites	GC/MS	38,39
Styrene	Mercapturic acids	LC/UV(fluorescence)	40-42
Butadiene	Mercapturic acids	LC(GC)/MS	43,44
Acrylamide	Metabolites	GC/MS	66

* LC Liquid Chromatography; GC Gas Chromatography; MS Mass Spectrometry; NPD Nitrogen phosphorus Detector; HS HeadSpace; SPME Solid Phase MicroExtraction

pattern of amino acid alkylation was dependent on the compound studied and could yield valuable information regarding the nature of the active electrophile.

Since than the formation of hemoglobin adducts with a variety of exogenous agents and the possibility of using hemoglobin adducts for biomonitoring purposes have been investigated (80-93).

Hemoglobin adducts dosimetry

Genotoxic agents that interact with hemoglobin molecules can bind to various nucleophilic groups within side chains of reactive aminoacids. Most of the biomonitoring methods based on the dosimetry of hemoglobin adducts involve the quantification of modified aminoacids released after protein hydrolysis. The latter can be carried out in different ways: total acid (or basic) hydrolysis, modified Edman degradation or digestion with specific enzymes.

The total acid hydrolysis of the protein implies drastic analysis conditions (HCl 6 M, 110 °C, overnight) (94); that is why labile adducts could result unstable leading to the underestimation of the actual modification percentage and mild hydrolysis conditions should be used (95).

The modified Edman degradation consists in the selective hydrolysis only of the alkylated N-terminal aminoacid (Valine) from the rest of the protein by using pentafluorophenylisothiocianate as specific reagent. The purified modified aminoacid is than analysed by gas chromatography-mass spectrometry (GC/MS) (13,14). In this case protein modifications are detectable only if they occur on the N-terminal amino acid. Nevertheless the application of protein hydrolysis and modified Edman degradation has been widely investigated and applied to a wide range of genotoxic agents and represents a valid comparative method for the validation of alternative procedures (96-109).

Enzymatic digestions carried out with specific enzymes such as trypsin, involve mild reaction conditions and do not give rise to alkylated aminoacids but to a mixture of unmodified and modified peptides with known molecular weights. Modified peptides can be detected by Liquid Chromatography/Electrospray Ionization- Mass Spectrometry (LC/ES-MS) on the base of the mass increment given by the presence of the modification. Enzymatic digestion produces peptides that contain the N-terminal aminoacid and also internal portions of hemoglobin aminoacidic sequence. Hence the quantification of alkylated peptides takes into account the eventual more reactivity of internal aminoacid residues.

The possibility of applying enzymatic digestion strategy requires preliminary studies on the reactivity of hemoglobin molecules towords different genotoxic agents. These studies were carried out by *in vitro* incubations of synthetic peptides or hemoglobin from non-exposed subjects with xenobiotics under analysis (110-117). The structural study of the obtained modified globins allows the identification of each aminoacidic modification site. Than the incubation is carried out with decreasing amount of xenobiotic in order to reflect low exposure levels, commonly found in workplaces. In this way only the more reactive site will be still alkylated and the peptide containing the modification results as the most suitable for the evaluation of professional exposure and risk assessment.

Once the structural characterization is completed and the best biomarker was chosen, the enzymatic digestion strategy involves the construction of calibration curves based on the use of alkylated and deuterated peptides as internal standards.

Recently, this new approach, based on the quantification of modified hemoglobin peptides, has been proposed and, in some cases successfully applied, for the biological

monitoring of workers occupationally exposed to different classes of toxicants such epichlorohydrin, methyl bromide and butadiene (118-120), putting in evidence t potentiality of this strategy.

Discussion

The biological monitoring of subjects occupationally exposed to genotoxic agents ca be carried out by using suitable biomarkers. Once it has been proved the correlation betwee the concentration of the toxicant present in the environment and within biological fluids, th toxicants itself or one of its main metabolite can be used as exposure indexes trhough th evaluation of the *internal dose*. On the other hand genotoxic xenobiotics react both wit critical target molecules (DNA) and with proteins, leading to adducts formation (47-60,80 93). The latter is supposed to be random with respect to cellular nucleophiles, and as consequence proteic and DNA adducts are produced simultaneously. Ehrenberg and co workers (7) showed that for several xenobiotics, the ratio between the amount of histidin hemoglobin adducts and N-7 guanidine DNA adducts was similar both *in vivo* and *in vitro* Because DNA adducts relate to the external dose, this means that also the formation o alkylated hemoglobin aminoacids is linearly related to the genotoxic agent dose. Once th precise ratio has been determined, the quantification of adducts between getoxic agents an biological macromolecules, including hemoglobin, allow the evaluation also of th *biological effective dose*, that is the amout of xenobiotic able to reach the target tissue as consequence of the biotransformation processes and of the individual susceptibility.

Table 2 summarizes different anlytical techniques adopted for the quantification o macromolecular adducts formed by various xenobiotics both with DNA and with hemoglobin.

An ideal biological monitoring procedure to evaluate genotoxic risk should measure adducts with DNA molecules of target tissue. Unfortunately the latter could be unknown, no easily to sample or not sufficiently available for adducts analysis. That is why adducts with hemoglobin of peripheral erythrocytes can be used as valid DNA surrogate. In fact hemoglobin presents many advantageous requirements: it is easy to sample in high amount (about 150 mg/ml in plasma) through a single blood taking; it forms stable adducts which are not subject to repair processes and turnover mechanisms, and finally, it has a long life time (approx. 120 days in humans) permitting the evaluation of chronic as well as accidental exposure. On the contrary, albumin, another potential DNA surrogate, is less abundant (about 42 mg/ml in plasma), with a higher turnover (19 days) and is involved in transport processes that could interfere with the analytical technique.

Hemoglobin adducts mainly derived from the alkylation of cysteine, histidine and N-terminal valine residues and they can be detected in different ways. According to the chemical nature of each toxicant, protein adducts could be stable or unstable in standard acid protein hydrolysis conditions followed by GC/MS analysis. In the case of unstable adducts, hydrolysis is carried out in mild conditions, according to the Tannenbaum procedure (95). Otherwise the Farmer methodology is followed (94). In both cases the main disadvantage of this technique lies in the difficulty of separating the modified aminoacid from an excess of 10^6 unmodified aminoacid residues present in the reaction mixture. In order to overcome this inconvenience, Tornqvist and co-workers (14) developed a modified Edman degradation that allows the selective hydrolysis of the alkylated N-terminal aminoacid. Than, it can be isolated from the rest of the protein and analysed by GC-MS. This methodology do not consider the influence of other aminoacid residues, within globin chains, eventually more reactive than the N-terminal one. The alkylation level of the N-terminal Valine of globin

Table 2. Macromolecular adducts dosimetry

Alkylating agents	Alkylated macromolecule	Analytical technique	References
Benzene	Hb	Hydrolysis	83-85,107,108
Styrene	DNA	^{32}P-post labelling/Radiolabelling	64,71/88
	Hb	Nickel-Raney/Radiolabelling	90/88
		N-alkyl-Edman modified	101
		Enzymatic digestion	109
Butadiene	DNA	^{32}P-post labelling/Hydrolysis	65/55-57
	Hb	N-alkyl-Edman modified	114
		Enzymatic digestion	120
Epichlorohydrin	DNA	^{32}P-post labelling	49
	Hb	N-alkyl-Edman modified	54,86,96
		Hydrolysis	54
		Enzymatic digestion	118
Ethylene oxide	DNA	Hydrolysis	52,69
	Hb	N-alkyl-Edman modified	52,106,117
		Hydrolysis	7
Methyl bromide	Hb	Hydrolysis	60,92,93
		N-alkyl-Edman modified	91
		Enzymatic digestion	112,116,119
Acrylamide	Hb	N-alkyl-Edman modified	66,103
		Hydrolysis	66,100,102
4-aminobiphenyle	DNA	Hydrolysis	67,68
	Hb	Hydrolysis	68,95,104

chains could represents only a minor fraction of the total number of adducts and, as a consequence, in the case of low levels exposure, the modified Edman degradation procedure is not be able to detect the actual level of protein adducts.

Alternatively, adducts hemoglobin dosimetry procedures could be based on the quantification of modified peptides rather than alkylated aminoacid residues. In this way the specific reactivity of the whole hemoglobin reactive sites towards genotoxic agents of different nature is taking into account. This analytical methodology requires the enzymatic digestion of modified hemoglobin molecules followed by a preliminary structural study of the protein, aimed to the identification of each modified peptide. Than, the peptide that is still alkylated even at low dose of xenobiotic agent, is chosen as the most accurate exposure biomarker and quantified by LC/ES-MS analysis.

Analysis of data literature on hemoglobin adducts as biomarkers to monitor exposure to genotoxic agents shows that the analytical approach based on the quantification of modified hemoglobin peptides can be used for biological monitoring purposes. The actual application of this methodology in the case of environmental carcinogenic substances widely used in industry (118-120), showed that the quantification of modified peptides could represent a new frontier in the definition of a more and more sensitive and accurate method for the biological monitoring of occupationally exposed workers.

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