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Short communication

Mass spectrometric analysis of haemoglobin adducts formed by methyl bromide in vitro

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Abstract

An analytical procedure is described for the identification of the adducts formed by interaction of methyl bromide and haemoglobin. The reaction products of in vitro incubation of haemoglobin with methyl bromide have been characterised by electrospray mass spectrometry and gas chromatography–mass spectrometry. A prominent reactivity of several potential nucleophilic sites of haemoglobin was observed. Analogous results were recorded on blood samples of workers exposed to methyl bromide. The results obtained represent the basis for the complete structural characterisation of the modified haemoglobin and demonstrate the usefulness of the proposed analytical approach for the evaluation of alkylation degree and the identification of modified amino acids in proteins.

1. Introduction

Methyl bromide (MeBr) is a toxic gas widely used as a fumigant of field soil for control of a large spectrum of pests and pathogenic organisms. MeBr is currently applied at rates of up to 125 g/m^2, the amount depending on the national regulations and agreement between grower and fumigator. Other major uses of MeBr include the fumigation of stored food in warehouses, of grain in silos and the disinfection of soil in greenhouses. Application is made by trained and licensed contractors because of the high toxicity of MeBr gas to humans and other mammals. Many accidental exposures to MeBr with lethal effects in humans have been reported [1,2]. People liable to be exposed include the applicators, other workers entering the area treated with MeBr and those living in the immediate neighbourhood of the fumigated areas.

Mutagenic properties of MeBr were previously demonstrated in bacteria and barley, and its alkylating properties are known [3–6]. MeBr is an alkylating agent with a low specific affinity toward sites in DNA critical for mutation induction, as compared to other alkylating agents [3,7]. MeBr reacts in vitro and in vivo with enzymes and proteins [8,9]; the cysteine-thiol group was

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suggested in several studies as the principal site of electrophilic reaction [10].

In this paper the authors report a study on the interaction between MeBr and haemoglobin (Hb) in vitro in order to assess the possibility to use the Hb adducts analysis as a biological index of exposure to MeBr. For a correct evaluation of the toxicological hazard, the knowledge of the reactivity of the single amino acids constitutive of Hb is needed. Such reactivity is not only dependent on the nature of the side chain, but also on its availability to react within the protein structure according to their tertiary structure. For this reason, mass spectrometry was extensively applied to the characterisation and quantification of modified amino acid within the polypeptide chain of Hb, combining the use of gas chromatography–mass spectrometry (GC–MS) and electrospray mass spectrometry (ES-MS); a prominent reactivity of several potential nucleophilic sites of Hb was observed, including cysteine, histidine and N-terminal valine.

2. Experimental

2.1. Materials

HPLC grade-solvents and reagents were obtained from Carlo Erba (Milano, Italy). A Vydac C_4 (214TP54, 25 × 0.46 cm, 5 μm) column was used for globin separation by HPLC.

Methylated amino acid standards were purchased from Sigma (St. Louis, MO, USA). MTBSTFA, N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide, was obtained from Pierce (Rockford, IL, USA).

An SPB-1 fused-silica capillary column [30 m, 0.25 mm I.D., 0.25 μm film thickness from Supelco (Bellefonte, PA, USA)] was used for gas chromatographic separation of amino acids.

2.2. In vitro alkylation

To prepare a solution of MeBr 5 m*M* for in vitro incubation, gaseous MeBr was bubbled in cold pure ethanol and the concentration of the solution was determined from the increase in weight, according to Iwasaki [11]. Red cells were washed three times with isotonic 0.9% NaCl solution and 2 ml of erythrocytes were suspended in 8 ml of 10 m*M* sodium phosphate, pH 7.0. Aliquots of ethanolic MeBr solution were added to 10 ml of red cell sample prepared as above. The reaction mixture was left at 37°C for 16 h, added to an isopropanol–HCl solution and centrifuged for 10 min at 2000 *g*.

2.3. Globin preparation

The globins from in vitro incubation and from the blood of workers exposed to MeBr were precipitated by addition of ethyl acetate; precipitated globins were collected by centrifugation at 3000 *g* for 30 min, washed with ethyl acetate and pentane and dried under a nitrogen stream.

2.4. HPLC separation of globin chains

Globin chains were purified by RP-HPLC using the procedure developed by Shelton et al. [12] with the following modifications: the samples (500 μg of globin dissolved in 100 μl TFA, trifluoracetic acid, 0.1% in water) were loaded onto a Vydac C4 large pore column equilibrated in the following buffers: (A) 80:20 water–acetonitrile, 0.1% TFA; (B) 40:60 water–acetonitrile, 0.1% TFA. The column was equilibrated at 52% of buffer B before loading the samples, then B was raised to 69% in 24 min. Before analysis, globin samples were filtered through a 0.45 μm filter (Millipore). For analytical runs 100-μg samples were used; for preparative runs, 1 mg was injected. The column effluent was monitored at 280 nm; protein fractions were manually collected and directly injected into the ES-MS source.

2.5. Electrospray mass spectrometry

ES-MS analysis of intact globin chains was performed by a BIO-Q triple-quadrupole mass spectrometer (VG, Manchester, UK). Samples from HPLC separation (10 μl, 50 pmol) were injected into the ion source at a flow-rate of 2

μl/min; the ES-mass spectra were scanned from 1400 to 600 u at a scan cycle of 10 s.

Mass scale calibration was carried out using myoglobin as reference compound. Quantitative analysis of components was performed by integration of the multiple charged ions of the single species.

2.6. GC–MS analysis

The amino acid analysis from hydrolysed globin chains was performed by a TRIO 2000 (Fisons, Manchester, UK) GC–MS apparatus equipped with a gas chromatograph 5890 Series II (Hewlett-Packard, Palo Alto, CA, USA). For the amino acids analysis 10 nmol of globins were hydrolysed with 300 μl HCl 6 *M* at 120°C for 18 h under vacuum; the dry samples were derivatised to *tert.*-butyldimethylsilyl (TBDMS)-derivatives with 40 μl of a mixture of acetonitrile–MTBSTFA (50:50, v/v) at 100°C for 2 h. The solution was diluted to 150 μl with acetonitrile and 0.5 μl were used for the analysis. The following analytical conditions were used: injector 260°C; ion source 180°C; GC–MS interface 260°C; GC column temperature raising from 60°C to 280°C at 3°C/min.

Mass spectrometric analysis was performed in scan mode from 50 to 600 u using a scan time of 0.6 s and an interscan time of 0.1 s.

3. Results and discussion

The reaction products after in vitro incubation of MeBr with Hb under different conditions have been characterised and blood samples of workers exposed to MeBr were analysed. The degree of modification of reactive sites on Hb was evaluated by ES-MS analysis, whereas the reactive amino acids within the polypeptide globin chains were identified by GC–MS analysis. A prominent reactivity of several nucleophilic sites of Hb was observed, including the N-terminal valine, cysteine and histidine.

Acid denatured globin chains from Hb were directly analysed by RP-HPLC after precipitation following the procedure described above. Protein fractions, corresponding to modified α- and β-chains were collected at around 21 and 31 min, respectively. These fractions were directly analysed by ES-MS to figure out the average methyl group number for each globin. A molecular mass of 16010.5 ± 2.6 was measured for the modified β-globin corresponding to the introduction of 10 methyl groups in the native protein chain (molecular mass 15867.2). As for the α-chain, a molecular mass of 15238.9 ± 4.1 was recorded (Fig. 1) with an increase of 112.5 mass units over the unmodified globin chain (15126.4), corresponding to the addition of 8 methyl groups. Only one main species was observed for each globin; less alkylated minor components failed to be detected possibly due to their lower concentration.

The same sample was submitted to acid hydrolysis and then amino acids were analysed by GC–MS (Fig. 2) as TBDMS-derivatives, in order to identify those residues within the polypeptide chains involved in the formation of the adducts. The results showed the presence of modifications on the imidazolic ring of histidine (both 1- and 3-methyl isomers), the thiol group of cysteine and the α-amino group of N-terminal valine in both globins. The identity of the amino acid adducts was established by comparison of the corresponding mass spectra with those of synthetic standards. As an example, in Fig. 3 the spectrum of the methyl cysteine from alkylated globin is reported in comparison with that of the respective standard. The relative abundance for each adduct was evaluated by using the synthetic methylated amino acids as reference external standard that allowed detection of 1.9 μmol of adduct/mmol of Hb. It must be observed that N-methyl valine and methyl cysteine can occur in vivo at three orders of magnitude lower as background [13,14]. The sensitivity level required can be achieved by using the same GC–MS approach working in the single ion monitoring (SIM) mode.

Both α- and β-globins showed the same reactivity either for the amino acids residues involved in the adduct formation and for the extent of modification. It must be observed, however, that for adducts which are labile in the conditions of

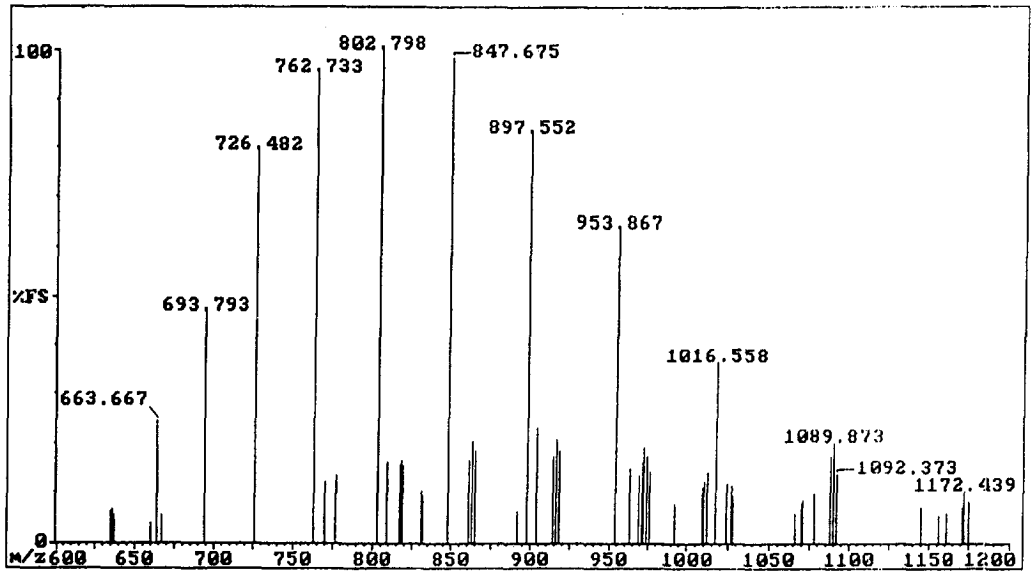

Fig. 1. ES-MS analysis of RP-HPLC fraction containing methylated β-globin showing the molecular mass at 15238.89 ± 4.14, corresponding to the introduction of 10 methyl group in the native β-globin chain.

acidic hydrolysis, the above GC–MS approach cannot be used for the identification of modified amino acids, such as methyl esters of carboxylic amino acids. These labile adducts can be analysed by using enzymatic hydrolysis [15].

By using the same analytical procedure the analysis of blood samples of workers professionally exposed to MeBr was performed (results will be reported elsewhere); the obtained results confirmed the pattern of adducts formation at

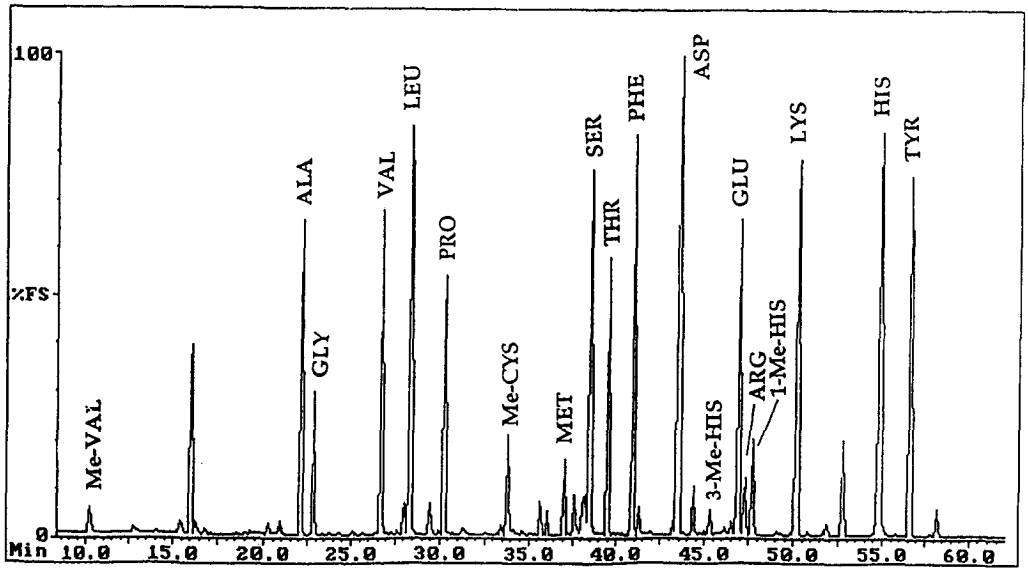

Fig. 2. Total ion current (TIC) of GC–MS analysis of TBDMS-amino acids obtained by acid hydrolysis of methylated globin sample.

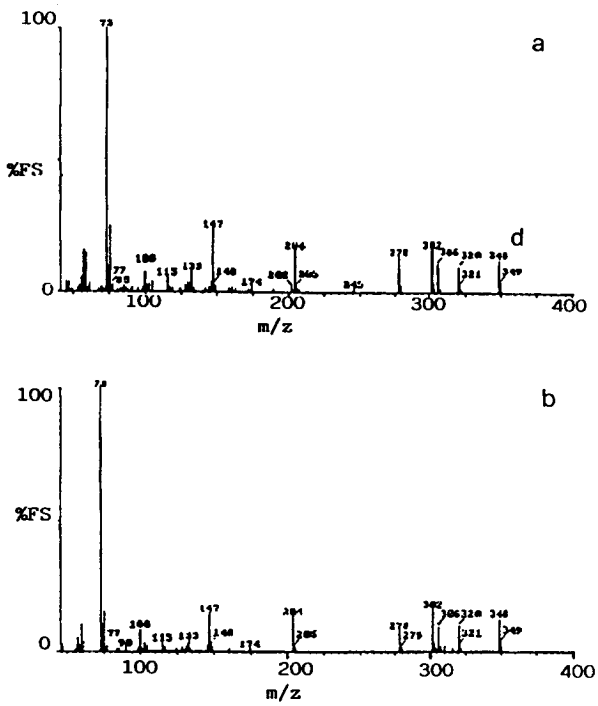

Fig. 3. Electron impact mass spectrum of methyl-cysteine from alkylated globin (a) compared to that of the synthetic standard (b).

level of the N-terminal valine as well as of other nucleophilic sites.

The results we are reporting show the useful-

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References

[1] A. Jaquet, Dtsch. Arch. Klin. Med., 71 (1901) 370.
[2] J.O. Greenberg, Ind. Med. Surg., 40 (1971) 27.
[3] L. Ehrenberg, S. Osterman-Golkar, D. Singh and U. Lundqvist, Radiat. Bot., 14(1974) 185.
[4] V.F. Simmon, K. Kauhanen and R.G. Tardiff, in D. Scott, B.A. Bridges and F.H. Sobel (Editors), Progress in Genetic Toxicology, Elsevier, Amsterdam, 1977, p. 249.
[5] G. Djalali-Behzad, S. Hussain, S. Osterman-Golkar and D. Segerback, Mutation Res., 84 (1981) 1.
[6] M. Moriya, T. Ohta, K. Watanabe, T. Miyazawa, K. Kato and Y. Shirasu, Mutation Res., 116 (1983) 185.
[7] E. Vogel and A.T. Natarajan, in F.J. de Serres and A. Hollaender (Editors), Chemical Mutagens, Plenum, New York, NY, 1982, Vol. 7, p. 259.
[8] K. Iwasaki, I. Ito and J. Kagawa, Ind. Health, 27 (1989) 181.