Arch Toxicol (1995) 69: 306–311

ORIGINAL INVESTIGATION

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Gas chromatography-mass spectrometry determination of ethylenethiourea hemoglobin adducts: a possible indicator of exposure to ethylene *bis* dithiocarbamate pesticides

Received: 18 July 1994/Accepted: 19 October 1994

Abstract Ethylene*bis*dithiocarbamates (EBDC) are an important class of fungicides used to control crop diseases and prevent mold. Ethylenethiourea (ETU), reported to be their main degradation and metabolic product in animals and man, may have teratogenic and carcinogenic properties. The feasibility of monitoring exposure to ETU on the basis of the formation of adducts to hemoglobin (Hb) was investigated. Rats given a single oral dose of ETU (from 62.5 to 500 mg/kg body wt) formed stable covalent ETU-Hb adducts. Mild acid hydrolysis of the protein regenerated ETU, allowing its detection by isotope dilution gas chromatography-mass spectrometry (GC-MS). The amount of released ETU increased with the dose. The dose-response curve fitted a linear model only between 62.5 mg/kg and 250 mg/kg. Acid-releasable ETU was also positively identified in the hemoglobin of workers exposed to Mancozeb, an EBDC formulation. In the exposed group, 40% had ETU-Hb adducts levels ranging from 0.5 to 1.42 pmol ETU/mg Hb. Such adducts might be useful for measuring EBDC exposure in humans.

Key words Adducts · Ethylenethiourea · Ethylene*bis*dithiocarbamate fungicides · Hemoglobin · Dosimetry

Introduction

Ethylene*bis*dithiocarbamate (EBDC) fungicides are widely used on fruits, vegetables, cereals and other food crops. They have a wide range of application, being highly effective against a broad spectrum of fungi and plant diseases (National Research Council 1987). EBDC are of regulatory concern because of ethylenethiourea (ETU), one of the metabolic products of their decomposition in mammals and plants. ETU may also be present as an impurity in these fungicides, the residues of which on crops may also be partly transformed into ETU during food processing (World Health Organization 1988).

ETU inhibits thyroid hormone production, is a rodent carcinogen (thyroid and liver) and has been classified as a potential human carcinogen (US Environmental Protection Agency 1989; Doerge and Takazawa 1990). Temporary alterations in central nervous system function and acute renal and transient heart failure have been reported after exposure to EBDC, even though the exposure conditions in these cases were not fully described (Koizumi et al. 1979; Israeli et al. 1983).

Human exposure to EBDC has been calculated on the basis of estimated consumption of dietary residues of ETU in treated crops. Upper and lower limit estimates of exposure to ETU were 3.65 and 0.24 μg/kg body weight per day, respectively.

EBDC and ETU enter the organism mainly through the respiratory tract (aerosol, dust), skin and mucous membranes (occupational exposure) and the digestive tract. Adverse effects on the thyroid gland and kidney have been reported among workers exposed to EBCD (Koizumi et al. 1979; Smith 1984). It has been suggested that occupational exposure to EBCD as Maneb and Mancozeb might be monitored by measuring ETU levels in urine of pesticide workers (Savolainen et al. 1989; Kurttio et al. 1990). The potential disadvantage of using urine samples as a biomarker is that the measurements of excreted metabolites often reflect recent exposure.

Current biomonitoring programs seek to assemble exposure information obtained from different analytical approaches, seeking correlations between exposure

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and biochemical or biological effects. A new form of dosimetry has been introduced in these biomonitoring investigations, based on quantitative analysis of the in vivo products of the reaction of a chemical carcinogen with protein.

Hemoglobin has been used as a suitable macromolecule for dose monitoring in man, for the following main reasons: large amounts are easily obtained from animals and man and stable adducts can be detected many weeks after acute exposure. Furthermore, in chronic exposure the covalent adduct can accumulate in hemoglobin molecules over their life-time (120 days in humans) (reviewed in Skipper and Tannenbaum 1990). Therefore, assay of hemoglobin adducts gives a more accurate estimate of the content in the body, representing the past and recent history of an individual's exposure to xenobiotics.

Protein adduct monitoring implies the use of very specific techniques, since levels of protein modification are in the range of pmol/g protein. Several methods, such as high performance liquid chromatography (HPLC) and gas chromatography (GC) coupled to different detectors, have been used for ETU trace analysis (Kurttio et al. 1988; Krause 1989), but these methods, while sensitive, often do not give the selectivity and specificity required, which can be achieved only with mass spectrometric determination.

The need to develop suitable methods for biological monitoring of exposure to EBDC prompted us to investigate whether ETU can interact with hemoglobin. This study set out to verify the formation of ETU-derived Hb adducts in rats in vivo and to set up a highly selective method for the quantitative analysis of ETU-Hb adducts. In addition, we attempted to measure adducts in the hemoglobin of a group of Italian workers, occupationally exposed to EBDC.

Material and methods

Chemicals

Ethylenethiourea (ETU) and dithiotreitol were obtained from Aldrich Chimica (Milano, Italy) and ethylene-d4-diamine hydrochloride from MSD-Isotopes (München, Germany). Carbon disulfide was obtained from J.T. Baker (New Jersey, USA). *N-tert* Butyl dimethylsilyl trifluoroacetamide (MTBSTFA) and 1-*tert* butyl dimethylsilyl imidazole (TBDMSIM) were purchased from Fluka (Buchs, Switzerland). Solvents were from Merck (Bracco, Italy). Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-PDH) were obtained from Boehringer Mannheim Biochemicals (Germany) and Sephadex G-25 from Pharmacia (Milano, Italy).

Deuterated ETU (ETU-d4) synthesis

Isotopically labelled ETU was synthesized following the method of Allen et al. (1955) with minor modifications. Briefly, 6.3 mg ethylene-d4-diamine hydrochloride, dissolved in 126 µl 0.88 M potassium hydroxide, 126 µl ethanol and 6.3 µl carbon disulfide were placed in a 1-ml vial. The solution was heated for 3 h at 60°C, 1 drop of concentrated HCl was added and the reaction temperature was raised to 90°C. After 7 h at 90°C, the solution was cooled and the crystalline product purified on preparative silica gel thin layer chromatography (solvent system was chloroform:methanol = 95:5).

We obtained 1.7 mg ETU-d4 (yield 36%) with an isotopic purity of 98% as checked by mass spectrometry.

Instrumentation and ETU quantification

Gas chromatographic and mass spectrometric data were obtained on a Hewlett-Packard 5890, series II gas chromatograph coupled to a Hewlett-Packard 5971 mass selective detector. The mass spectrometer was operated in the electron impact (EI) mode with the following conditions: calibration with standard autotune routine, ion source temperature 200°C. Gas chromatographic conditions were as follows: WCOT fused silica capillary column CP Sil 5 CB 25 m × 0.32 mm i.d., film thickness 0.12 µm (Chrompack, Cernusco S/N, Italy), and oven temperature kept at 120°C for 1 min, then increased to 240°C at 15°C/min and held there for 5 min. The injector temperature was 240°C and the carrier gas (He) head pressure was 15 kPa. Analyses were performed in the splitless mode.

ETU was analyzed by high resolution gas chromatography-mass spectrometry with selected ion recording (HRGC-SIR).

ETU was quantitated as its TBDMS derivative by an isotope dilution method, using ETU-d4 as internal standard. Samples were derivatized at room temperature (20 min reaction) using a derivatisation mixture consisting of 50% acetonitrile and 50% MTBSTFA containing 20% TBDMSI. The samples were analyzed by monitoring the most intense fragments: ions m/z 217, 273, 315 for ETU and ions m/z 221, 277, 319 for ETU-d4. A calibration curve was obtained by analyzing 2 µl of mixtures containing different amounts of ETU (0.4–4 pmol) plus a constant amount of ETU-d4 (4 pmol) as their TBDMS derivatives.

In vitro studies

Two assays were performed to assess the binding of ETU to human Hb: 330 mg human Hb from lysed fresh red blood cells (RBC) was incubated with 1.5 mM ETU in the absence or presence of non-induced rat liver microsomes (0.4 mg protein/ml incubation medium). Both reactions were carried out in 5 ml 0.1 M phosphate buffer, pH 7.4.

In the microsomal incubation, reaction was started by adding an NADPH generating system (NADP, 3 µmol; G-6-P, 177 µmol; G-6-PDH, 7 U/sample). The samples were incubated at 37°C for 1 h in the dark and incubations were terminated by chilling in ice. The Hb solution was separated from microsomes by centrifugation at 105 000 *g* for 30 min.

The adducted hemolysates recovered from the assays were processed as described below.

Animal treatment and isolation of rat hemoglobin

Male CD rats (200 ± 10 g bodt wt) were purchased from Charles River (Calco, Como, Italy). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985).

Upon arrival, the rats were housed at 22°C temperature, 50% relative humidity, with a 12-h light/dark cycle, for a week before use.

Four groups of rats were given single oral doses of ETU dissolved in 0.25% carboxymethyl cellulose. The first group received 62.5 mg ETU/kg body wt, the second 125 mg, the third 250 mg and the fourth 500 mg. Control rats were given the vehicle alone.

The animals were anesthetized with ether and killed 24 h after dosing. Each animal's blood was collected into heparinized tubes. Fresh blood was separated into plasma and RBC by centrifugation (5 min at 3000 *g*). The RBC were rinsed three times with equal volumes of 0.9% NaCl solution and then lysed with 4 vol 0.7 M phosphate buffer, pH 6.5. The hemolysate was centrifuged at 10000 *g* for 20 min to remove cellular debris and the volume was reduced by filtering the solution through a YM-10 (10 kDa cut off) Amicon membrane in a 50-ml Amicon stirring cell under N2 pressure. The Hb was then separated from low-molecular-weight material (e.g. GSH adducts, free ETU or metabolites) by Sephadex G-25 chromatography. Hb solution, 5 ml, containing about 600 mg Hb, was eluted with water through a $3 \times 30\ cm^2$ column. The red Hb band was collected. The volume was reduced to about 6 ml using the Amicon ultrafiltration system described above. The nearly pure Hb solution was cooled at 4°C and added dropwise to 600 ml acid acetone (0.015% HCl) maintained at < -10°C with stirring. The precipitated globin was pelleted by centrifugation, washed with cold acetone, dried and weighed.

The adducts were isolated by acid treatment of 300 mg globin dissolved in 10 ml 0.1 N HCl in a glass tube. The hydrolysis was carried at 60°C for 4 h. Before incubation, each sample (controls included) was spiked with an equal amount of ETU-d4, as internal standard. The hydrolysate was neutralized with 3 N NaOH and extracted three times with 2 vol ethyl acetate. The combined organic extracts were dried under a nitrogen stream and derivatized as described above.

Validation of the method by human hemoglobin samples

Human blood samples were kindly provided by Professor M. Maroni, International Centre for Pesticide Safety, Milano, Italy.

Twenty-three male subjects worked in a small factory, where EBDC was produced. Fifteen workers were employed in the synthesis of Mancozeb, a polymeric complex of EBDC manganase with zinc salt. The control group consisted of eight males who worked elsewhere in the same factory and who had no known exposure to EBDC. All 23 subjects underwent a complete medical examination.

The distribution of subjects in the study, by age, tobacco and alcohol consumption is shown in Table 1. None of the subjects was receiving any pharmacological treatment.

Occupational exposure to Mancozeb occurred by inhaling the powder dispersed in the working area. Mancozeb air concentrations were measured on the same day of biological sampling, by means of a personal sampler, at a flow rate of 3.6 l/min. The average concentration in air was 1 mg Mancozeb/m^3 for the exposed group (M. Maroni, personal communication).

Blood samples were collected into heparinized tubes at the end of an 8-h work shift. Informed consent of the donors was previously obtained. RBC were immediately separated from plasma by centrifugation. The red cells were then washed four times with 0.9% NaCl solution and lysed with 2 vol distilled water. Samples were processed as described for rat blood samples.

Table 1 Distribution of subjects in the study groups by age, tobacco consumption and alcohol intake

	Exposed ($n = 15$) %	Controls ($n = 8$) %
Age		
30–40 years	73	50
> 45 years	27	50
Non-Smokers	40	62
Alcohol intake		
No alcohol	40	–
⩽ 50 g/day	47	100
⩾ 50 g/day	13	–

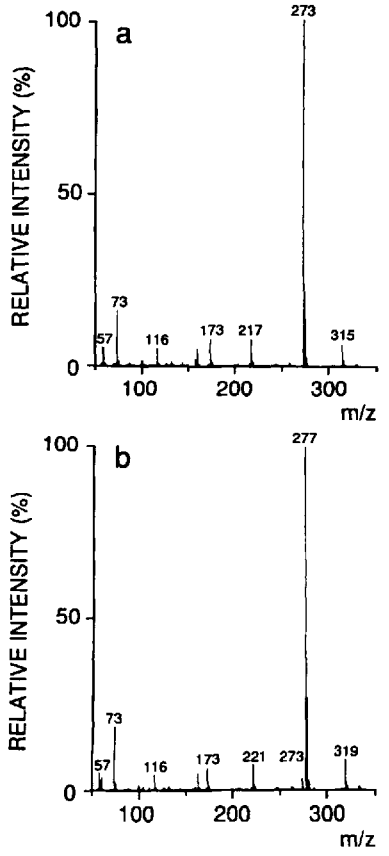

Fig. 1 Mass spectra of *tert* butyl-dimethylsilyl derivatives of ETU (**a**) and ETU-d4 (**b**). Common to all EI spectra of TBDMS derivatives is the loss of the TB group, which gives the most intense peak (M-57)

Results

Mass spectra of derivatized ETU and ETU-d4 standards showed major fragment ions at 273 and 277, respectively, corresponding to loss of the *tert* butyl moiety (Fig. 1).

Figure 2 shows typical GC-SIR chromatograms obtained by injecting ETU standard solutions and rat Hb samples. A 2% contribution of ETU-d0 on m/z 273 was present in the standard solution of newly synthesized ETU-d4. Therefore, quantitative calculations were corrected for this. The contribution of ETU-d4 on m/z 273, represented by the peak area ETU-d0/ETU-d4 ratio,

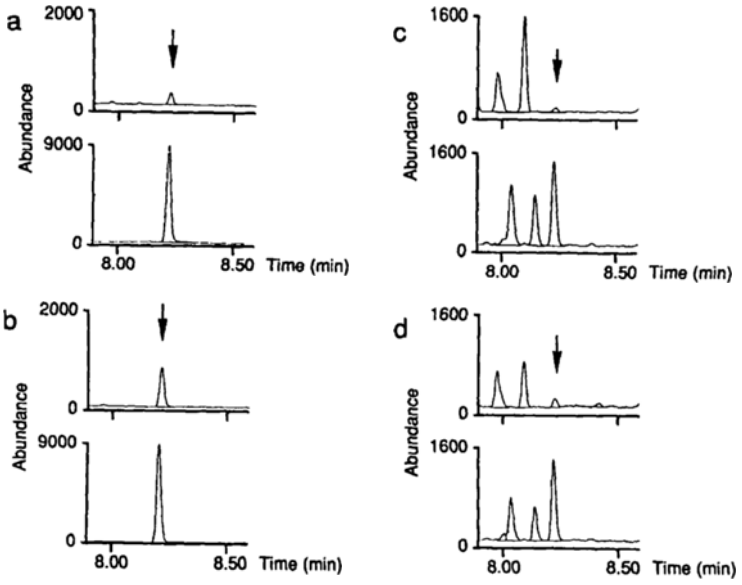

Fig. 2a–d Typical HRGC-SIR analysis of *tert* butyldimethylsilyl derivatives of ETU. The *upper trace* was produced by monitoring at m/z 273 (ETU), the *lower trace* was produced by monitoring at m/z 277 (ETU-d4, internal standard). **a** ETU-d4 (2 ng injected); **b** ETU standard (80 pg + 2 ng ETU-d4 injected); **c** ethyl acetate extract from control rat globin; **d** ETU extracted from treated rat globin. Peak indicated with an *arrow* is ETU. GC conditions were as follows: oven temperature 120°C (1 min) to 240°C at 15°C/min; injector temperature 280°C, in splitless mode

was subtracted to the same ratio calculated in rat and human samples.

Calibration curves obtained by analyzing increasing amounts of ETU and a constant amount of ETU-d4 showed a linear relationship between the ratio of the peak area of ETU and the area of the internal standard. The correlation coefficient was 0.99.

The detection limit for ETU in SIR analyses was $\leqslant 30$ fmol/mg rat Hb and $\leqslant 500$ fmol/mg human Hb. The human Hb samples showed an unexpectedly higher background that affected the limit of sensitivity.

We detected ETU released from acid hydrolysis treatment of the globin samples. The clean-up of hemolysate on the Sephadex G-25 column was efficient at separating the protein from unbound ETU, which was not recovered along with the Hb fraction when aliquots of control Hb were spiked with ETU. Extensive extractions of unhydrolysed globin with organic solvents resulted in no ETU being detectable, suggesting that the ETU measured after hydrolysis of the protein derives from covalently bound ETU adducts, sensitive to acid treatment.

The identity of the analyte was confirmed by comparing its retention time and ion ratios to those of the ETU standards. The ETU in the hydrolyzed globin was quantitated by comparison with the internal standard ETU-d4, as described in Materials and methods.

Of the ETU incubated with human Hb, 0.0009% was covalently bound to the protein in the absence of any enzymatic system (0.2 pmol ETU/mg Hb). The presence of rat liver microsomes and the NADPH generating system increased the binding of ETU to human Hb 14 times (3 pmol ETU/mg Hb), suggesting that microsomal monooxygenases are necessary to produce highly reactive species.

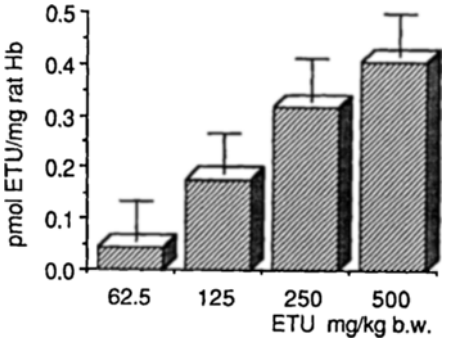

Fig. 3 Levels of adducts formed with Hb of rats 24 h after single oral doses of ETU. Bars are means ± SE (n = at least 3). Analysis of variance was done using Duncan's one-way multiple comparison test. There was no difference between the doses of 250 and 500 mg ETU/kg body wt. For the other doses, the differences were significant at $p \leqslant 0.05$ or better

The amounts of released ETU detected in the rat globin samples are shown in Fig. 3 and are reported in terms of the amount (pmol) of ETU released per mg Hb hydrolyzed. Rats given different acute doses of ETU had significantly different amounts of ETU-Hb adducts.

The values of acid-releasable ETU increased with the dose, and the dose-response curve was found to fit a linear model only between 62.5 and 250 mg ETU/kg body wt (Fig. 4). Within this linear portion, the efficiency of Hb adduct formation was 0.115 (pmol ETU/mg Hb)/(mmol ETU/kg body wt). At doses higher than 250 mg ETU/kg body wt, the amounts of released ETU were not linearly related to the dose.

Acid-releasable ETU was also positively identified in the Hb of workers exposed to Mancozeb concentrations as high as 1 mg/m^3 (Table 2). In the exposed

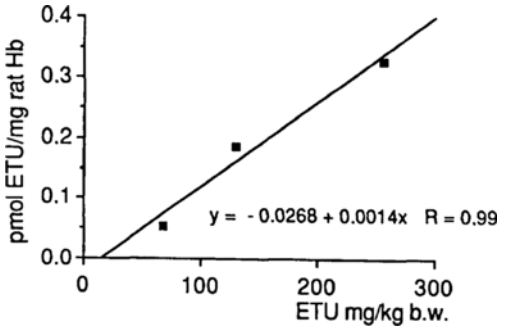

Fig. 4 Linear regression fit of the data within the linear portion of the dose-response curve

Table 2 ETU-Hb adducts in Italian workers occupationally exposed to Mancozeb. The subjects were all males. Isolation of adduct was achieved by acid treatment of the human globin. The hydrolysates were extracted with ethyl acetate. The organic extracts were dried, derivatized and analyzed by HRGC-SIR

Sample	ETU adducts (pmol/mg Hb)
Control group	
C1	< 0.5[a]
C2	< 0.5
C3	< 0.5
C4	< 0.5
C5	< 0.5
C6	< 0.5
C7	< 0.5
C8	< 0.5
Exposed group[b]	
E1	0.7
E2	< 0.5
E3	0.73
E4	< 0.5
E5	< 0.5
E6	< 0.5
E7	< 0.5
E8	1.42
E9	0.94
E10	< 0.5
E11	0.5
E12	< 0.5
E13	0.5
E14	< 0.5
E15	< 0.5

[a]The limit of detection was $\leqslant$ 0.5 pmol ETU/mg Hb
[b]The subjects were exposed to average air concentration of 1 mg Mancozeb/m^3

group, 40% had higher adduct levels than controls, ranging from 0.5 pmol to 1.42 pmol/mg Hb.

Discussion

Mass spectrometric analyses of ETU residues have been conducted mostly in food crops and waters (Doerge and Miles 1991; Van der Poll et al. 1993) and never applied to biological matrices.

We have developed a gas chromatographic-mass spectrometric method for the biomonitoring of low levels of ETU in blood samples. The use of ETU-d4 as internal standard has improved the accuracy of our measurements.

Our results show that ETU and/or its reactive intermediate bind to Hb of both rats and humans, to form an acid-sensitive adduct. The overall mechanism by which such adducts are formed in vivo is not known, but it is likely to be similar to the initial step believed to take place in the formation of the covalent binding of bioactivated ETU and microsomal proteins (Decker and Doerge 1991). Oxidation of ETU by FMO and/or P-450 systems may result in the formation of a sulfenic acid, a highly reactive electrophile. The reaction of an ETU-derived sulfenic acid with the Hb-cysteine sulphydryl might then result in a disulfide retaining both the imidazoline ring and the sulfur moiety. The mild acid treatment during the globin processing would reduce the disulfide bonds between oxidized ETU and cysteine, resulting in the complete release of bound ETU.

In order to gain information on the chemistry of the ETU-Hb adduct, we reacted globin from control and ETU-treated rats with 50 mM dithiothreitol. Samples were extensively washed to remove any excess of dithiothreitol and released ETU, then the globin samples were subjected to mild acid hydrolysis and ETU was measured in the hydrolysate as described in Materials and methods. No ETU was detected in any sample (data not shown), suggesting that the cysteine residue on rat Hb is the major site of ETU adduction.

Calculation based on the measured binding of ETU to rat Hb showed that, within this dose range, binding was approximately one to two molecules of compound for every 10^5 molecules of Hb.

Though Hb binding sites for ETU reactive intermediates were still available, the non-linear formation of ETU-Hb adducts at doses above 250 mg ETU/kg body wt (2 mmol ETU/kg body wt) may be related to saturation of the ETU metabolism. Numerous processes governing the fate of xenobiotics (such as absorption, plasma protein binding and excretion) do in fact become saturated, at doses up to 10^{-4}–10^{-3} mol/kg in rodents (Neumann 1984).

Reactive metabolites generated from ETU by either FMO or P-450 are preferentially trapped by endogenous GSH and very little remains available for interaction with cellular targets (Decker and Doerge 1991). Therefore the formation of ETU-Hb adducts may form only at very high doses, such as those we used.

Since possible deviation from linearity down to dose zero would constitute a basis for a threshold, and the ETU doses we used would be rarely encountered in biomonitoring, our main concern was the adequacy of available technology to measure the low levels of ETU

adducts arising from possible exposure in humans. ETU-Hb adducts were individually analysed in our laboratory in workers exposed to EBDC formulations, and our preliminary results on human Hb analysis showed detectable levels in blood samples obtained from workers exposed to Mancozeb. In the exposed population, 40% showed values ranging from 0.5 to 1.42 pmol ETU/mg Hb. Since our subjects have been exposed to relatively similar concentration of Mancozeb in the air, the variability in adducts levels might be due to individual differences. No correlations were found with age, smoking habits and alcohol intake; therefore, different explanations can be offered for the higher levels of ETU found in some workers:

1) Different working habits (care in wearing protective clothes) with the consequence of variability in Mancozeb uptake.
2) Personal hygiene.
3) Genetic heterogeneity in metabolic activation and detoxification.

The amounts of adducts measured in human samples ranged from 3 to 9 molecules ETU for every 10^5 molecules of Hb, higher than in our animal model. This was unexpected, considering that the reactivity of cysteine in human Hb is two orders of magnitude lower than in rat Hb (Neumann 1984; Hutchins et al. 1988) and that the exposure levels in our subjects were low. Knowing that the Mancozeb air concentration was about 1 mg/m^3 and assuming the breathing rate to be 0.2 l/min per kg for 8 h of light-moderate workload, we can estimate that the amount of Mancozeb inhaled was 20% of the acceptable daily intake for EBDC (ADI 500 µg/kg body wt per day).

These observations imply that either a different metabolism or different sites of adduction might favor formation of ETU-Hb adducts in humans. The exposure regimen itself can dramatically affect both the accumulation and removal of adduct and adduct levels are also dependent on the time since the last exposure.

We have limited information about our human subjects' past occupational exposure to EBDC. Their blood samples were taken 4 weeks after they started Mancozeb production. The effects of intermittent exposure or exposure for less than the lifetime of the RBC are still poorly understood in Hb adduct dosimetry (Granath et al. 1992).

Too few human blood samples were analyzed to arrive at any conclusion and no attempt can be made to describe the dose-response pattern in humans. However, it did prove feasible to measure ETU-Hb adducts in humans exposed to EBDC levels below the ADI. The highly specific and sensitive method described in this paper may therefore have potential application in risk assessment associated with human exposure to EBDC as well as in the analytical determination of ETU residues in various matrices.

Acknowledgements The authors wish to thank J. Baggott and the G. A. Pfeiffer Memorial Library who helped prepare the manuscript.

This research was supported by CNR Grant Targeted Project "Prevention and control disease factors — Subproject Environmental quality." Contract No. 9200232.PF41.

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