Hydroxyethylvaline adduct formation in haemoglobin as a biological monitor of cigarette smoke intake

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Abstract. The ethylene oxide adduct formed on the N-terminal valine in haemoglobin was investigated as a biological monitor of tobacco smoke intake. The modified method developed for the determination of the hydroxyethylvaline adduct (HOEtVal) involved reaction of globin with pentafluorophenyl isothiocyanate, extraction of the HOEtVal thiohydantoin product, derivatization of this by trimethylsilylation and quantitation by capillary gas chromatography with selective ion monitoring mass spectrometry using a tetradeuterated internal standard. The method was applied to globin samples from 26 habitual cigarette smokers and 24 non-smokers. There was a significant correlation between cigarette smoke intake as measured by the average number of cigarettes smoked per day and HOEtVal levels (r = 0.537, p < 0.01). Background levels were found in non-smokers (mean 49.9 pmol/g Hb, range 22-106 pmol/g Hb). Smoking increased these levels by 71 pmol/g Hb/10 cigarettes per day.

Cotinine levels in plasma of the smokers were determined by GC-NPD using 2-methyl-4-nitroaniline as internal standard. For non-smokers cotinine was determined by GC-NPD using 2-methyl-4-nitroaniline as internal standard. There was no correlation between number of cigarettes smoked per day and cotinine levels (r = 0.297, p > 0.05) although cotinine was correlated with HOEtVal (r = 0.43, p < 0.01).

The HOEtVal adduct levels thus appear to be a suitable biomonitor for exposure to hydroxyethylating agents in cigarette smoke, reflecting an integrated dose over the erythrocyte lifetime. This is in contrast to plasma cotinine determinations which reflect only the previous day’s exposure to nicotine in smoke.

Key words: Hydroxyethylvaline — Haemoglobin — Cigarette smoke — Biological monitoring — Cotinine — carcinogen adduct

Introduction

Cigarette smoking has long been known to be associated with an increased risk of developing bronchial neoplasia (Doll and Hill 1950). Since then, there has been a need for a means to assess the exposure to cigarette smoke as well as a means of quantifying this risk. This requirement has been further emphasized by epidemiological reports indicating that exposure to environmental tobacco smoke (“passive smoking”) is associated with a genotoxic risk (IARC Monograph 1986; Wald et al. 1986; Remmer 1987 and references therein).

The markers that have been mainly used up to now for monitoring exposure to tobacco smoke have included carbon monoxide, determined as carboxyhaemoglobin in blood, thiocyanate in plasma and urine and cotinine (a major metabolite of nicotine), in saliva, plasma and urine. Of these, cotinine levels in either plasma or urine are generally accepted as the best parameters to distinguish smokers from non-smokers, as these markers show very low background levels caused by sources other than tobacco smoke (Jarvis et al. 1984; Pojer et al. 1984; Muranaka et al. 1988).

To acquire data on the carcinogenic risk associated with smoking (which cannot be accurately achieved by cotinine measurements) determination of the biologically effective dose of the genotoxic chemicals present within the individual is required. As these compounds are believed to initiate cancer via the formation of covalent linkages with DNA, the measurement of such adducts would be a valid indicator of biologically effective dose. Carcinogen-DNA adducts have been determined in smokers by immunoassay procedures, or by post-labelling with radioactive phosphorus (a technique that may now achieve a sensitivity of one altered base per 10¹⁶ nucleotides) (Farmer et al. 1987). The detection of DNA adducts by chemical techniques, such as HPLC or GC-MS, would require the availability of large amounts of DNA which is not normally possible. As an alternative approach, one may assay the extent of carcinogen adducts with blood-based proteins. One such protein which is extremely abundant is the globin of the haemoglobin molecule, and adducts with this protein have been used to monitor exposure to a variety of environmental and occupational carcinogens (Neumann 1984; Bailey et al. 1987; Farmer et al. 1987). Initially this work, which was pioneered by Ehrenberg and collaborators, (Ehrenberg et al. 1974) involved the analysis of carcinogen-modified cysteine and histidine residues within the protein chain. More recently, analytical developments have greatly enhanced the sensitivity of protein adduct analysis, allowing studies to be made of carcinogen exposure from tobacco smoke.

The classes of compounds to which these developments have been applied are the aromatic amines and al-
phatic epoxides. In the former case Bryant et al. (1987) demonstrated that mild alkaline hydrolysis could be used to liberate aromatic amines that were covalently bound as the sulphonic acid amide to cysteine residues within haemoglobin. Analysis of these amines by negative ion chemical ionization GC-MS showed that, for example, the sulfynamide bound adduct of 4-aminobiphenyl was present to a mean level of 154 pg/g Hb (±47) in smokers compared to 28 pg/g Hb (±13) in non-smokers. A method for the analysis of adducts of ethylene oxide with haemoglobin was developed by Törnqvist et al. (1986a) using pentafluorophenyl isothiocyanate to cleave the alkylated N-terminal valine adduct from the protein by a modified Edman degradation. The product, which was a thiohydantoin derivative, can readily be extracted and analysed by GC-MS. The average levels of the N-(2-hydroxyethyl) valine (HOEtVal) adduct were found to be increased from a concentration of 58±25 pmol/g Hb in non-smokers to 389±138 pmol/g Hb in smokers of more than 20 cigarettes per day (Törnqvist et al. 1986b). No dose-response relationships were reported in either of these studies. In this paper we describe a modification of this approach of Törnqvist et al. (1986a, b) for the determination of HOEtVal in haemoglobin which we have used to monitor the biologically effective dose of ethylene oxide received by the erythrocytes of habitual smokers, non-smokers and non-smokers acutely exposed to five cigarettes. Methods developed for the estimation of plasma cotinine are also described, and the results from these analyses were compared with the HOEtVal levels.

Materials and methods

Cotinine was purchased from Sigma (Poole, UK) and 2-methyl-4-nitroaniline from Aldrich Chemical Co. Ltd (Gillingham, UK). The purity of these two compounds was determined by capillary gas chromatography with flame ionization detection (GC-FID). d4-Methylnorcotinine was synthesized by a previously described method (Daenens et al. 1985). The product was purified by preparative silica gel thin-layer chromatography using the solvent system ethyl acetate-methanol-ammonium hydroxide 70:25:5 and analysed by capillary GC-MS. Pentfluorophenyl isothiocyanate (Fluka, Glossop, UK) and N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma) were each purified by vacuum distillation. Ethylene oxide was obtained from Fluka and d4-ethylene oxide from MSD Isotopes (Montreal, Canada). HOEtVal and hydroxypropylvaline (HOPrVal) were synthesized by the method of Törnqvist et al. (1986a). All solvents were BDH (Poole, UK) Analar grade, except for formamide which was of Biochemical grade. Ether, toluene and acetonitrile were redistilled before use. Formamide was purified by passage through a column containing aluminium oxide (Woelm, activity grade l). All other solvents were used without further purification. The glassware used in the extraction and derivatization of plasma and haemoglobin samples was chromic acid washed and silanized with a 5% solution of dimethyldichlorosilane in toluene. The coated fused silica capillary columns used for the GC and GC-MS analyses were prepared in the author’s laboratory (MRC Toxicology Unit).

Subjects. Blood was collected in heparinized tubes from habitual cigarette smokers (n = 26) and from a group of non-smokers (n = 24). The habit of the smokers was established and regular. When possible, subjects were asked to collect and count their cigarette stubs over a 3-day period to ascertain their average daily cigarette consumption. The study was performed at the end of the collection period. Each subject attended at 10.00 hours on the study day, when 10 ml blood was taken from a forearm vein.

For studies of acute cigarette exposure three apparently healthy, non-smoking individuals were recruited from the staff of the Hammersmith Hospital. Each subject attended at 10.00 hours, and 10 ml blood was taken from a forearm vein. During the next hour five cigarettes were smoked, and at the end of this time a further 10 ml blood was taken for adduct determination. After a further 1 and 22 h 10 ml blood was again taken for adduct determination. Plasma and erythrocytes were separated by centrifugation at 900 g for 10 min. Plasma was used for the determination of cotinine levels and globin isolated from the erythrocytes for quantifying levels of HOEtVal adduct.

Synthesis of internal standard, globin alkylated with d4-ethylene oxide. d4-Ethylene oxide was added to heparinized fresh human blood collected from a non-smoking individual. The blood (10 ml) was incubated at 37°C overnight on a shaking water bath and the red blood cells then separated by centrifugation at 900 g for 10 min. The cells were washed twice with isotonic saline (3 volumes) and then lysed by adding 1 volume of water. Cell debris was removed by centrifugation (20000 g) and the globin precipitated from the supernatant with 1% HCl in acetone. The protein was washed with 1% HCl in acetone, acetone and then ether. The globin was finally dried in a vacuum desiccator overnight. Globin alkylated with unlabelled ethylene oxide was prepared in a similar manner.

Quantitation of the unlabelled and deuterium labelled HOEtVal adduct in the synthesized alkylated globin samples was determined by GC-FID. Globin was hydrolysed in 6 M HCl in vacuo at 110°C for 24 h and the amino acids in the protein hydrolysates esterified by reaction with 3 M HCl/n-butanol and then acylated by derivatization with heptafluorobutyric anhydride (Bailey et al. 1980). Quantitation by GC was made by reference to an internal standard of HOPrVal added to the globin samples after the hydrolysis step.

Determination of hydroxyethylvaline adduct in haemoglobin. Analyses were carried out using 50 mg amounts of globin isolated from blood as described above. The analytical procedure used was essentially that developed by Törnqvist et al. (1986a, b), with modifications to improve the specificity and sensitivity of the method. Briefly the assay involves the following steps:

1. Globin sample (50 mg) dissolved in formamide (1.5 ml).
2. Internal standard added (globin alkylated with d4-ethylene oxide containing 72.3 pmol d4-HOEtVal).
3. Sample reacted by shaking overnight at room temperature and then for 90 min at 40°C with pentfluorophenyl isothiocyanate (7 μl).
4. Hydroxyethylvaline - pentfluorophenylthiodyantoin (HOEtVal-PFPTH) derivative extracted into ether.
5. Dried extract redissolved in toluene and the solvent extract washed with water and 0.1 M Na2CO3.
6. Dried toluene extract reacted for 30 min at 60°C with 40 μl acetonitrile: bistrimethylsilyltrifluoroacetamide (1:1 v/v).