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Investigation of protein-styrene oxide adducts as a molecular biomarker of human exposed to styrene

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Abstract: Hemoglobin-styrene oxide adducts in blood have been studied as a molecular biomarker of worker exposed to styrene. Determination of protein-styrene oxide adducts in different biological samples with modified Raney-Ni procedure is described in this paper. The following biological samples have been investigated: fresh rat blood reacted with styrene oxide in vitro; rat blood reacted with styrene or styrene oxide in vivo; vein blood from workers exposed to styrene in two factories. The data showed that there was a good linear dose-response relationship between reacting dose of styrene oxide or styrene and amount of protein-styrene oxide adducts in both in vitro and in vivo experiments. For human samples, a dose-response relationship between protein adducts and styrene exposure can be found in glass fiber factory, but not in piano manufacture plant.

Keywords: protein adduct; styrene oxide; molecular biomarker

Introduction

Biomarker, defined as cellular, biochemical or molecular alterations that are measurable in biological media, such as human tissues, cells, or fluids, have been used by generations of epidemiologists in their research. Molecular biology and laboratory technology have shown an exponential growth in the last decades. Today biomarker can be identified down to a level of less than one alteration in 10^6 DNA bases. Molecular biomarkers may be internal indicators of exposure to external xenobiotics (chemical, physical, and biological), they may reflect early, subclinical adverse health effects, or they may define the innate susceptibility of the human host. Adducts are stable complexes of reactive chemicals (originated in environment or endogenous), and cellular macromolecules (DNA, RNA or protein) that contain one or more covalent bonds between the two moieties. DNA adducts are thought to play a major role in carcinogenesis because covalent bonds are formed between the chemical and one or more DNA strands in adducts formation. DNA adducts become a kind of important molecular biomarkers in researches on environmental chemical exposures and risk assessment strategies in recent years. Although protein adducts themselves play no direct role in tumor induction, for some chemicals there are data that correlate protein adducts with other processes more directly involved in carcinogenesis (Goldring, 1990). Research findings indicate that, for some carcinogens, determination of the amount of protein adducts can give estimates of the DNA adduct burden. Proteins (hemoglobin and albumin) are easy to obtain and protein adducts represent logger-term exposures. Since human albumin (Alb) has a half-life of 20 – 25 days and human hemoglobin (Hb) has a life-span of 120 days, stable adducts of either protein are representative of exposures occurring over the 1 – 4 month period prior to blood collection.

Up to now, the formation of protein adducts with a number of chemicals have been done in animal studies. But studies in human field exposure to chemicals are still very few. For example, protein adducts with chemicals such as ethylene oxide, propylene oxide, trinitrotoluene have been used as biomarkers in monitoring human risk and exposure to those chemicals (Liu, 1995). We focus on protein adducts with styrene and their metabolites styrene oxide in this study. Researches have shown that styrene is not seriously toxic, but their oxide metabolites are potential mutagens and carcinogens (Ponomarkov, 1984). The mechanism may be the DNA adducts formation with styrene oxide. Animal studies show that the protein adducts have been formed and have the corresponding response relationship (Jin, 1997). Christakopoulos *et al*. found that there are relationships between the hemoglobin adducts with styrene oxide in blood samples from workers in glass fiber factory by modified Edman method (Christakopoulos, 1993). But Karen C Y *et al*. found that there was not any relationship between styrene exposure dose and globin adducts with styrene oxide in blood samples from boat factory by Ra-Ni method (Yeowell, 1996). It shows that the experimental results from field research with different methods have given dramatical differences. More researches should be done to find out the reasons of the difference. In this study, the modified Ra-Ni analytical method has been used to determine protein styrene oxide adducts in the blood samples collected from workers exposed to styrene with low concentration. The results show that there is a good linear dose-response relation between low concentration of styrene and hemoglobin-styrene oxide adducts in blood samples. The results also suggests that protein-styrene oxide adducts could be used as a potential molecule biomarker for human exposed to low concentration of styrene.

1 Materials and instruments

1.1 Chemicals

Styrene (99%); styrene oxide (SO – 97%); 3-phenylpropanol (3-PP); Raney-Ni (50% slurry in water); N-

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bromosuccinimide(NBS); 4-methystyrene; 4-methyl-2-phenyl ethanol (4-m-2PE); phenyl ethanol (2-PE); pyridine (anhydrous) and pentafluorobenzyl chloride (PFB-chloride) were obtained from Aldrich Chemical. Co., protease XIV, human hemoglobin (Hb), human albumin (Alb) were obtained from Sigma Co., Other chemicals were obtained from Beijing Chemical Reagent Co.

1.2 Preparation and purification of standard samples

(1) 4-methyl styrene oxide (4m-SO): 4m-styrene, NBS and water were stirred vigorously at room temperature for about 1h, the organic layer was collected and treated with NaOH for 2h at 60℃, 4m-SO was dried with anhydrous Na_2SO_4, and purified with a gel column by eluting with a mixture of methanol, methylene chloride and hexane (2:5:4). The structure of the product was confirmed by EI-MS (Rappaport, 1993). (2) Protein adduct of 4-methylstyrene oxide (4m-SO-Hb, 4m-SO-Alb): Hb (or Alb) was incubated with 4m-SO for 10h at 37℃, the purified protein (through column containing Sephadex G-25) was precipitated with cold acetone containing HCl and washed with acetone and dried to constant weight in a vacuum oven at 40-45℃.

The adduct was identified using a procedure described by Ting D W *et al*. (Ting, 1990).

1.3 Instrument

Gas-chromatograph (GC) 5890(HP), GC/MS 5989(HP), High speed centrifuge(Beckman), UV-spectrophotometer (HP) and vacuum oven (China).

2 Experimental method

2.1 Preparation of adduct sample

2.1.1 Modification of blood in vitro

Fresh whole blood from SD rats was modified with SO in vitro as follows: SO (0 - 7.5 mmol/L) in 0.5 ml saline was added to 1.2 ml blood to a final concentration of 0, 10, 30, 100, and 300 μmol/L and incubated in a water bath for 2h at 37℃ with occasional shaking.

2.1.2 Animal experiment

Styrene or SO in corn oil was administered up to SD rats at the following dosage levels: 0, 0.5, 1.0 and 3.0 mmol/L styrene/kg body weight and 0, 0.1, 0.3 and 1.0 mmol/L SO/kg body weight. The animals were anesthetized with methoxyfluorane 24h following administration, and the blood was collected by cardiac puncture with a heparinized syringe.

2.1.3 Human blood sampling

Human subjects consisted of 11 workers in glass fiber factory and 10 workers in piano manufacture plant. Individual exposure to styrene were measured by GC method (GB/T 16053 - 1995). Blood sampling was conducted on the day after measurement of exposure to styrene. Blood (5 ml) was collected into heparinized tubes and was refrigerated for up to 6h before processing.

2.1.4 Isolation of hemoglobin and albumen

Samples of whole blood were centrifuged to separate red cells. The red cells were washed with NaCl (0.15 mol/L) and water, and were centrifuged at 15000 r/min at 4℃ for 40 min. The Hb solution was added in dialysing membranes and was dialysed at 4℃ for 24h. Globin was precipitated by adding cold acetone containing HCl, and dried at 40 - 50℃ to constant weight. Alb was isolated as following: Saturated ammonium chloride solution was added to the plasma and centrifuged at 15000 r/min at 4℃ for 40 min. The Alb solution was penetrated by dialysing membranes and was precipitated by adding ammonium acetate solution, and dried to constant weight.

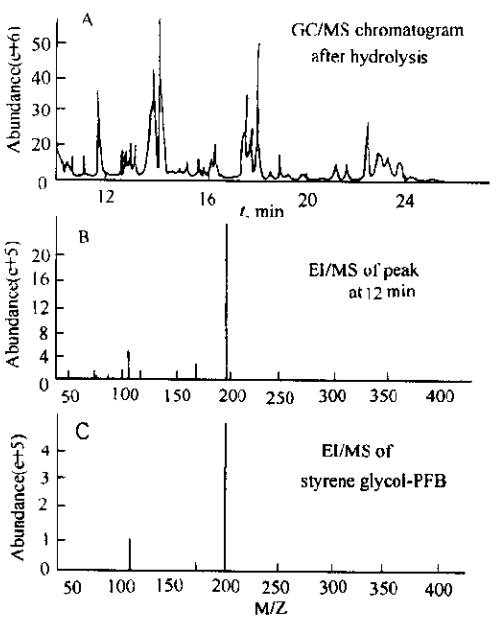

Fig.1 GC/MS/EI analysis of Hb-SO adducts in vitro

2.2 Analysis of protein adducts

2.2.1 Digestion of protein samples

Purified Hb or Alb 10 mg and 25 μg 4m-SO-Hb standards were dissolved in 4 ml water, 25 μg d8-SO-Hb, 1 ml 0.1 mol/L tris-HCl, and 750 μg protease XIV were added. The protein samples were digested at 37℃ for 4h, and were extracted twice with 5 ml ethyl ether, the aqueous phase was collected.

2.2.2 Ra-Ni procedure

To the aqueous solution were added Ra-Ni (6 times the weight of Hb), and 5 μg 3-pp, and let react at 4℃ for 40 min. Samples were extracted with 5 ml ether twice. Extracts were reduced to almost dryness under N_2.

2.2.3 Derivatization

The dried samples were dissolved in 1 ml hexane, 3 μl of pyridine and 1.5 μl of PFB-chloride were added to the samples which were derivatied at 50℃ for 20 min. Then samples were extracted, purified and dry.

2.2.4 GC/MS analysis

The derivatized samples were analyzed by GC/MS. A

capillary column (DB-5, 30m, 0.242 mm i.d, 1-μm phase thickness) was used, Methane was used as the chemical ionization reagent gas, carrier gas was He. The temperature programming was set at 75℃ for 2 min, then increased at 50℃/min to 200℃, which was held for 12 min, and then continue increased at 50℃/min to 250℃, which was held for 10 min. The temperature of the ion source was 150℃ and the temperature of injection port was 250℃ with splitless mode. The instrument was operated in the selected ion monitoring mode and focused at m/z 316 (2-PE-PFB), m/z 324 (1-PE-PFB, d8-2-PE-PFB), m/z 330 (4m-2-PE-PFB, 3-PP-PFB). Quantitation of adducts was based on peak area. Retention times for the adducts were as follows: 1-PE-PFB = 14.05 min, 2-PE-PFB = 15.47 min, d8-2-PE-PFB 15.38 min, 4m-2-PE-PFB = 17.62 min, 3-pp-PFB = 18.20 min.

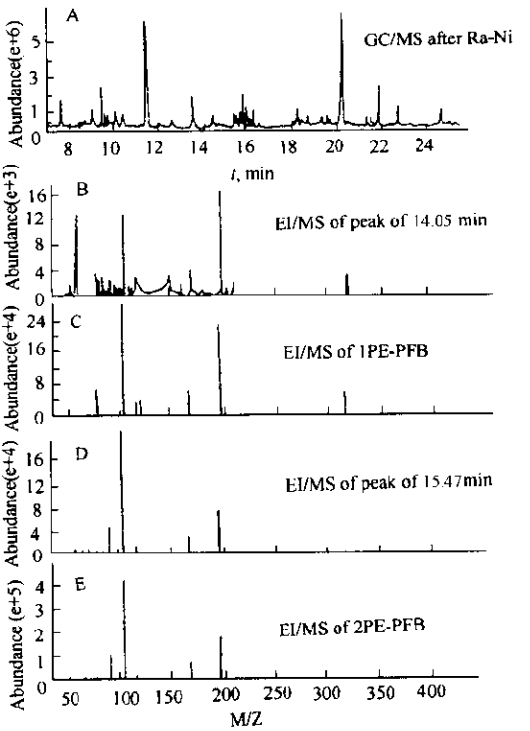

Fig.2 GC/MS/EI analysis of Hb-SO adducts(Ra-Ni) in vitro

3 Results

3.1 Protein-SO adducts in vitro

3.1.1 Analysis and identification of Hb-SO adducts

Protein cystine residues from rat whole blood was modified with 300 μmol/L SO in vitro. The three Hb-SO adducts: SO-carboxylic acid adducts (SC) and SO-cystine adducts including two isomers (1-PE, 2-PE), were detected with this procedure, Fig.1 and Fig.2 show the GC/MS total ionization chromatogram and the EI mass spectrum of the peak showing the same GC retention time as the standards. The adduct SG was identified as its pentafluorobenzoate derivative (Fig. 1) and the 1-PE, 2-PE adducts were identified as its pentafluorobenzoate derivative (Fig.2).

3.1.2 Relationship between protein-SO adducts and concentration of SO

For the modified rat blood with SO in vitro, a good relationship between the Hb-SO, Alb-SO adducts and concentration of SO is shown in Fig.3. The quantitative analysis of the three adducts showed 2-PE > 1-PE > SG.

3.1.3 The lowest limit of detection of analysis

Using 10 mg of samples and 0.1 ml of final samples solution, the concentration of lowest detectable adducts was found to

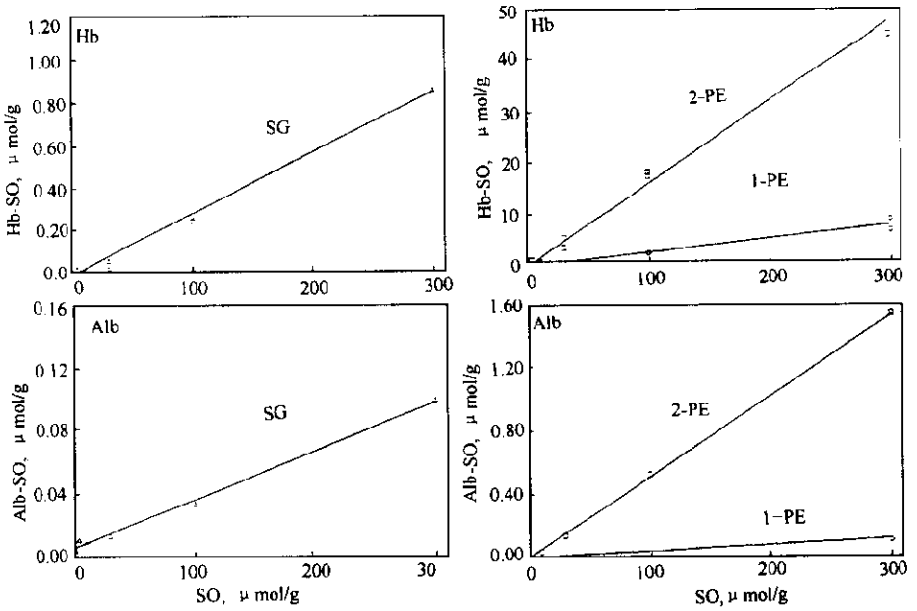

Fig.3 Protein-SO adducts in rat blood treated with SO in vitro

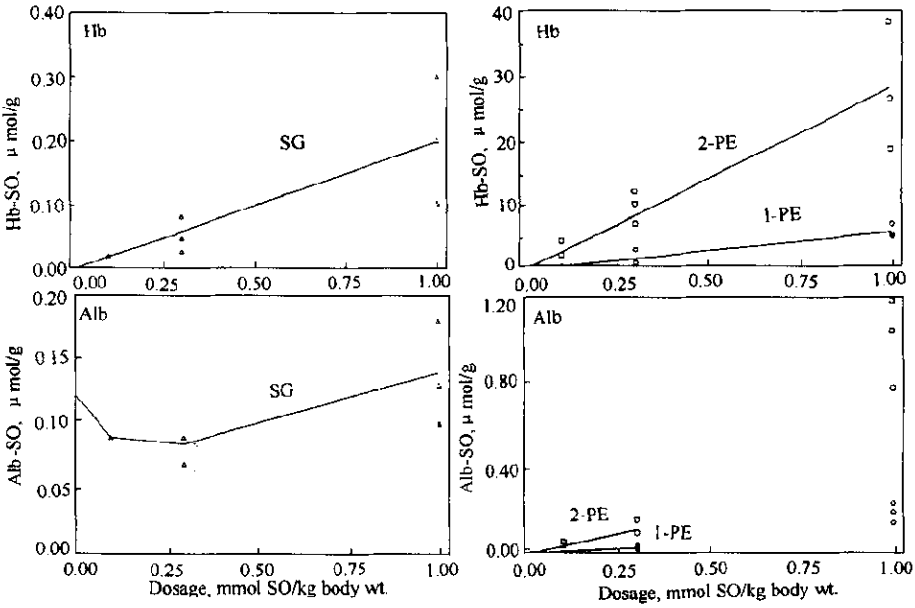

Fig.4 Protein-SO adducts in rat blood treated with SO in vivo

be 8 pmol/g protein for SG, 5 pmol/g protein for 1-PE and 0.6 pmol/g protein for 2-PE. The relative standard deviation for 30 analysis was 12%.

3.2 Protein-SO adducts in vivo

Fig.4 and Fig.5 show the dose-response relationship for Hb-SO and Alb-SO adducts as the three analyses following administration of SO and styrene to rat. The quantitative analysis of adducts shows 2-PE > 1-PE > SG (Fig.3 - 5). Similar data showed that there is a good linear relationship between protein adducts and reacting dose of SO or styrene in both in vitro and in vivo experiments.

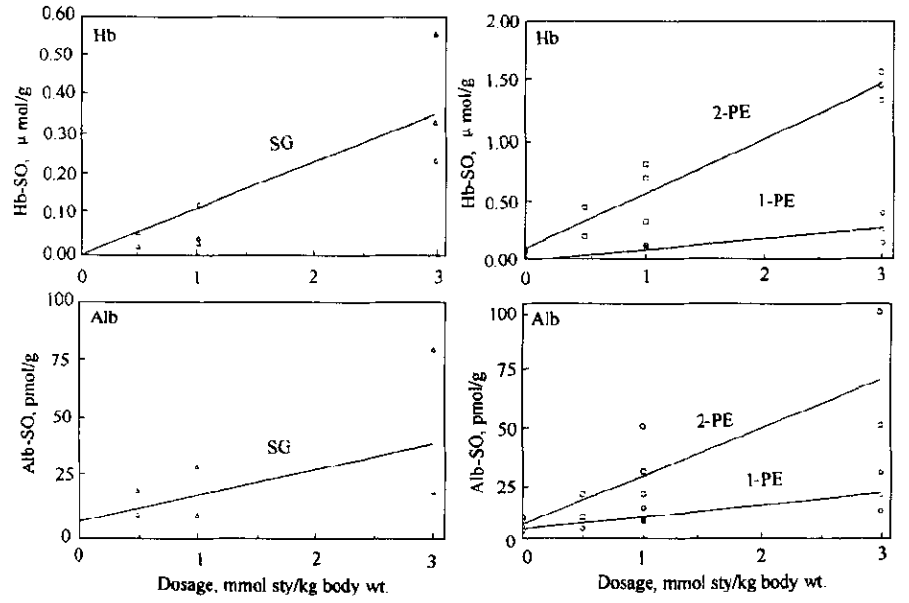

Fig.5 Protein-SO adducts in rat blood treated with styrene in vivo

3.3 Protein-SO adducts in human blood samples exposed to styrene

3.3.1 Detection of protein adducts in workers blood samples exposed to styrene in glass fiber factory

Exposure concentrations of styrene in glass fiber factory are listed in Table 1.

GC/MS analysis of protein-SO adducts is given in Fig.6. The relationship between the concentration of styrene and protein-SO adducts is given in Table 2, Fig.7 and Fig.8.

Table 1 Exposure concentration of styrene in glass fiber factory 1

Subject No.	Sex (F, M)	Age, years	Working age, years	Smoking age, years	Concentration of styrene, mg/m³		
					1st time	2nd time	Average
1	F	33	10		77.6	74.3	75.95
2	F	32	10		38.7	48.2	43.45
3	F	28	10		68.5	71.2	69.9
4	F	32	4		32.8	35.5	34.1
5	M	22	0.3	1	1.8	2.0	1.9
6	M	33	10	10	10.5	10.5	10.5
7	M	46	13		13.3	13.3	13.4
8	F	40	12		4.2	4.2	4.2
9	M	30	10	8	12.5	12.5	12.5
10	M	29	10	10	3.7	3.7	3.7
11	M	47	6	no 10	3.5	3.5	3.5

Note: volume of blood sample was 5 ml for every worker

Table 2 Protein-SO adducts in human exposed to styrene

Subject No.	Styrene[1], mg/m³	Adduct 1-PE[2], nmol/gGb	Adduct 2-PE[2], nmol/gGb	Adduct 1 PE[3], nmol/gAlb	Adduct 2 - PE[3], nmol/gAlb
1	75.95	–	4.00	0.00	3.01
2	43.45	0.07	3.05	0.00	2.18
3	69.9	0.38	3.77	0.00	2.86
4	34.15	0.37	2.84	0.01	2.24
5	1.9	0.47	3.66	0.05	3.00
6	10.5	0.30	2.58	0.30	1.92
7	13.4	0.08	2.09	0.00	3.61
8	4.2	0.26	2.37	0.00	1.99
9	12.5	0.24	2.38	0.41	2.79
10	3.7	0.13	2.12	0.18	1.66
11	3.5	0.21	2.30	0.00	1.81

Notes: 1. average of 2 determinations; 2. Hb-SO adduct; 3. Alb-SO adduct

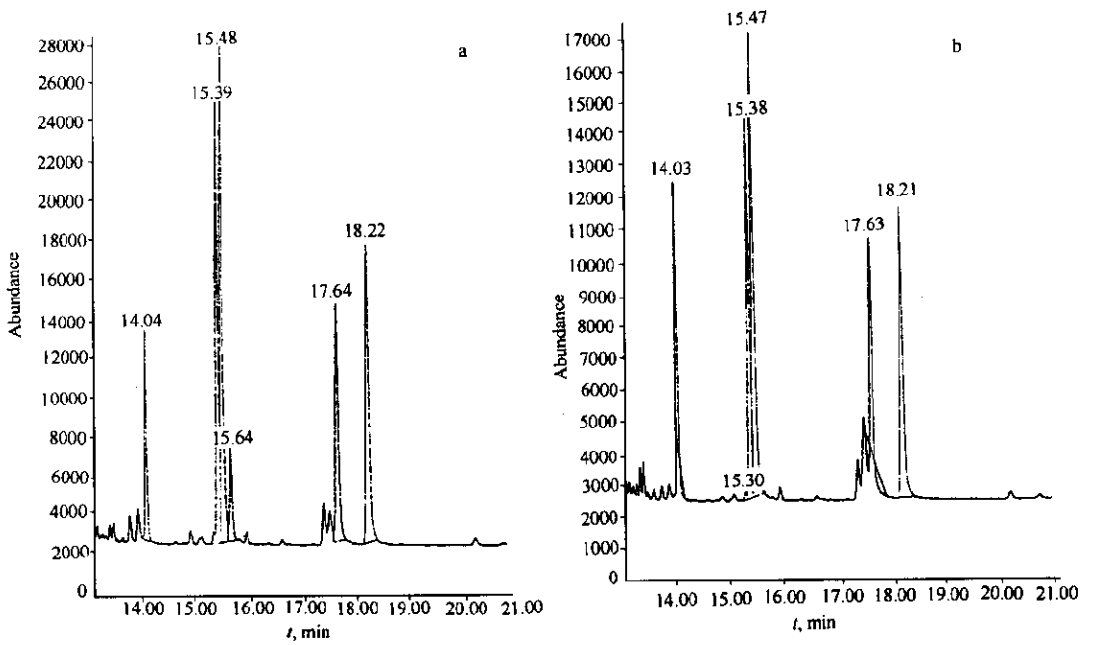

Fig.6 GC/MS analysis of Hb-SO adducts in human blood exposed to styrene
a: control; b: human blood

The results in Fig.7, 8 and Table 2 showed that: Hb-SO adduct > Alb-SO adduct in each sample, adduct 2-PE > 1-PE in both Hb and Alb. There is a good dose relation between hemoglobin-styrene oxide adduct (2-PE) and human exposure concentration of styrene (Fig 7). The data of the linear relationship is: $Y = 0.0190X + 2.3618$ ($r^2 = 0.5561$, $n = 11$), $Y = 0.0257X + 2.1075$ ($r^2 = 9433$, $n = 10$). There is not dose-relationship between protein adducts (1-PE in Hb, 1-PE and 2-PE in Alb) and the worker exposure concentration of styrene.

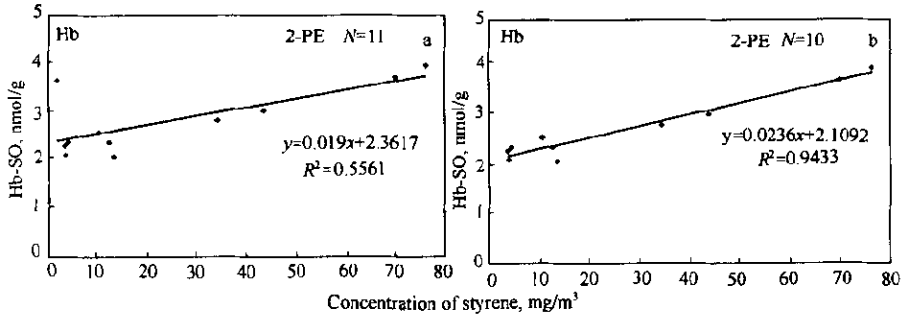

Fig.7 Hb-SO adducts (2-PE) in human blood exposed to styrene

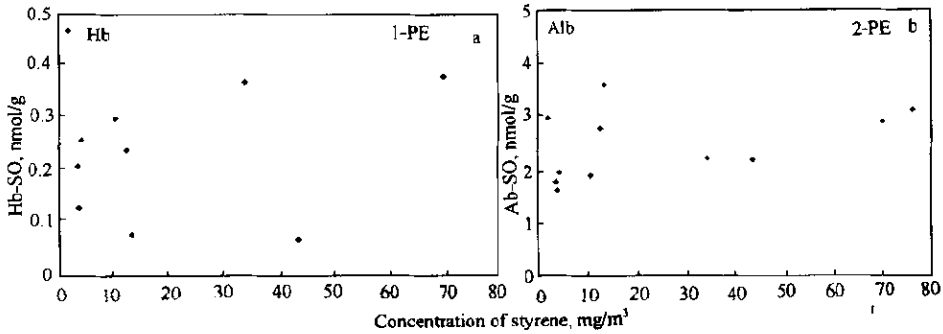

Fig.8 Protein-SO adducts in human blood exposed to styrene

3.3.2 Detection of protein-SO adducts in blood exposed to styrene in piano factory

Worker exposure concentration of styrene in piano factory are listed in Table 3.

Table 3 Worker exposure concentration of styrene*

Subject No.	Sex (F,M)	Age, years	Working age, years	Smoking age, years	Concentration of styrene, mg/m³		
					1st time	2nd time	Average
12	F	45	8	–	86.4	83.3	84.8
13	M	50	10	25	302.0	302.0	302.0
14	F	30	5	–	476.0	535.0	505.0
15	F	40	16	–	19.0	20.4	19.7
16	M	40	0.1	20	289.0	298.0	293.5
17	M	24	6	–	151.0	111.0	131.0
18	F	33	12	–	185.0	234.0	209.5
19	F	39		–	247.0	279.0	263.0
20	M	22	4	4	90.2	78.9	84.5
21	M	32	4	–	81.1	84.1	82.6

* . volume of blood sample is 5 ml for every worker

GC/MS analysis of protein-SO adducts. The relationship between the concentration of styrene and protein-SO adducts is given in Table 4. Fig.9 and Fig.10.

The results (Fig.9, Fig.10, Table 2) showed that adduct 2-PE > 1-PE (Hb and Alb). There was a weak relationship between hemoglobin-styrene oxide adduct (2-PE) and the worker exposure concentration of styrene. There was not relationship between other adducts of protein with styrene oxide and the worker exposure concentration of styrene.

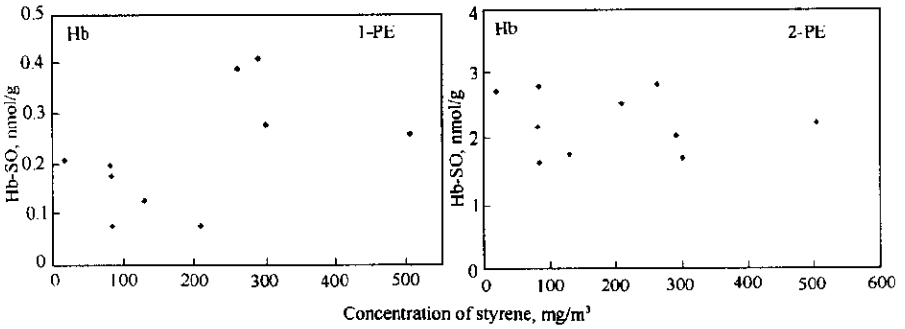

Fig.9 Hb-SO adducts(1-PE,2-PE) in human blood exposed to styrene

Table 4 Protein-SO adducts in human exposed to styrene

Subject No.	Styrene[1], mg/m³	Adduct 1-PE[2], nmol/gGb	Adduct 2-PE[2], nmol/gGb	Adduct 1-PE[3], nmol/gAlb	Adduct 2-PE[3], nmol/gAlb
12	84.8	0.08	1.64	0.00	2.32
13	302.0	0.28	1.70	0.14	2.96
14	505.6	0.26	2.24	0.00	1.42
15	19.7	0.37	2.74	0.00	2.48
16	293.5	0.41	2.05	0.00	2.70
17	131.0	0.13	1.77	0.00	1.93
18	209.5	0.08	2.54	0.00	1.79
19	263.0	0.39	2.84	0.00	1.43
20	84.5	0.18	2.81	0.00	2.01
21	82.6	0.20	2.19	0.00	2.83

1. average of 2 determinations; 2. Hb-SO adduct;
3. Alb-SO adduct

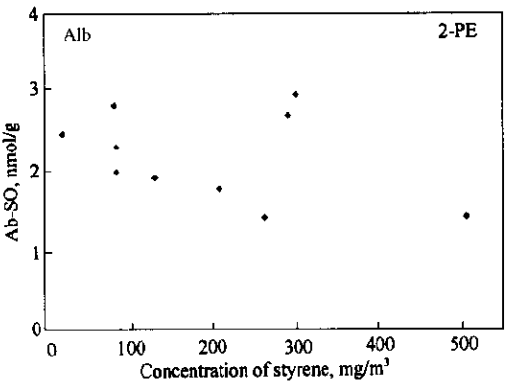

Fig.10 Albumin-SO adducts (2-PE) in human blood exposed to styrene

4 Conclusion and discussion

The results showed that the method for determination of protein adducts with styrene oxide by Ra-Ni was of high sensitivity (the lowest detectable adduct concentration was 0.6 pmol/g protein for adduct 2-PE) and good repeatability (the relative standard deviation for 30 analyse was 12% in vitro). Three protein-styrene oxide adducts (SG 1-PE 2-PE) can be detected in the same sample by this analytical procedure, and the amount of the three adducts was 2-PE > 1-PE > SG in both in vitro and in vivo. A dose-response relationship was also found between the adduct 2-PE (in Hb) with the concentration of workers exposed to styrene in the glass fiber factory, but none in piano manufacture plant.

This is the first report that there is a dose-response relationship between hemoglobin -SO(2-PE) adduct with exposure to low concentration of styrene in field studies. The results also suggest that protein-styrene oxide adducts could be used as a potential molecule biomarker for human exposed to low concentration of styrene. But this result has no statistical significance. More studies are required such as modification of the analytical procedure for increasing the sensitivity and repeatability of method, more field studies for statistical significance and so on.

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