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Investigation of the Adduct Formation between Styrene or Styrene Metabolites and Hemoglobin or Blood Proteins in Rats and Mice (In Vitro and In Vivo)

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Report

Investigation of the adduct formation between Styrene or Styrene metabolites and hemoglobin or blood proteins in rats and mice (in vitro and in vivo)

Study performed for the
ECETOC Task Force Styrene

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SUMMARY

This report describes experiments in which the possible adduct formation between styrene (S) or styrene 7,8-oxide (SO) and hemoglobin or plasma proteins in rats and mice was investigated.

The results of these experiments show that hemoglobin and plasma protein adducts are formed and can be detected following i.p. administration of S or SO to rodents. Appropriate methods were developed for the determination of the N-terminal valine adduct (hydroxyphenethylvaline). This adduct was found to be stable with time but in contrast adducts involving carboxyl groups were found to be unstable. Dose-response relationships were established for both rats and mice. Comparison between species showed that at low doses (below 0.5 mmol/kg body weight) the formation of N-terminal valine adducts is approximately 3 times greater in mouse than in rats. This approach with further refinement and verification by inhalation route may provide the basis for the development of a biological monitoring method. The total binding to plasma proteins is considerably higher than the binding to hemoglobin. The possibility of measuring adducts to plasma proteins should be explored and might possibly provide a more sensitive method for human monitoring.

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1. GENERAL

1.1. PURPOSE OF THE STUDY

The aim of the study is to investigate the possible adduct formation between styrene (S) or styrene-7,8-oxide (SO) and hemoglobin or other blood proteins in rats and mice following i.p. administration of S or SO and the feasibility of using this adduct formation for the development of a biological monitoring method.

1.2. DESIGN OF THE STUDY

The study design comprises the following approaches:

- Characterization of the spectrum of reaction products (adducts) in hemoglobin and plasma proteins. (Which are the reactive metabolites, which adducts are formed, and how much?)
- The structural identification of products by gas chromatography/mass spectroscopy (GC/MS).
- Determination of the stability of quantitatively important adducts. In biological systems this includes the chemical stability and biological half-life.
- Study of the dose-response relationship for the formation of adducts using a specially developed GC/MS method.

2. MATERIAL AND METHODS

2.1. CHEMICALS AND SYNTHESIS OF REFERENCE COMPOUNDS

2.1.1. Chemicals

[^{3}H]Styrene 7,8-oxide (3.88 GBq/mmol = 105 mCi/mmol, radiochemical purity 96.5%) and 7-[^{14}C]styrene (3.95 MBq/mmol = 107 µCi/mmol, radiochemical purity 98%) were obtained from the Radiochemical Centre, Amersham, UK. ($^2H_8$)Styrene (98 atom % 2H) was obtained from Sigma, St. Louis, MO. Unlabelled styrene 7,8-oxide (97%) was from Aldrich, Milwaukee, WI, and unlabelled styrene was from Fluka, Buchs, Switzerland. The purity of the compounds was checked by HPLC prior to use. Styrene was specifically checked for styrene oxide contamination. The concentration of styrene oxide was undetectable (below 0.1%) in unlabelled styrene and below 0.5% in radiolabelled styrene.

Formamide (analytical grade, Merck, D-W6100 Darmstadt, FRG) was extracted with pentane before use. Other reagents and solvents were of analytical grade and used without further purification.

2.1.2. Synthesis of ($^2H_8$)styrene 7,8-oxide

Deuterium-substituted styrene 7,8-oxide was prepared using a modification of the procedure described by KOLOGRIVOVA et al. (1980). ($^2H_8$)Styrene (0.5 g) was dissolved in 2.5 ml ethanol in a 25 ml round-bottomed reaction vessel. Sodium tungstate (Na_2WO_4), 75 mg, was added and the reaction mixture was heated under vigorous stirring at 60 to 65^{0}C (oil bath) for 30 min. The pH of the reaction mixture was then adjusted to 7.5 (0.1 M NaOH in EtOH) and 2 ml of 30% hydrogen peroxide (H_2O_2) was added through a dropping funnel during 40 min. The reaction mixture was kept at 65^{0}C with stirring for 12 hours. Three ml of water (pH 7.5) was added and the reaction mixture was shaken twice with 5 ml methylene chloride (CH_2Cl_2). The organic fractions were pooled and dried with anhydrous sodium sulfate. The solvent was then removed.

Abbreviations: GC - gas chromatography; MS - mass spectrometry; PFPITC - pentafluorophenyl isothiocyanate; PFPTH - pentafluorophenylthiohydantoin; NCI - negative chemical ionization; EI - electron impact; TLC - thin-layer chromatography; HPLC - high performance liquid chromatography. Hydroxyphenethylvaline is used as collective name for diastereomers of β-hydroxyphenetyl- and, possibly, α-(hydroxymethyl)benzyl-valine.

The residue was dissolved in 0.5 ml toluene: ethyl acetate (8 : 2) and applied to a column, 80 × 0.6 cm, packed with silica gel 60. The column was eluted with toluene: ethyl acetate (8 : 2). The styrene oxide-containing fractions were collected and evaporated. The yield was 130 mg material.

For further purification 30 mg of the synthetic product was dissolved in 15 ml hexane and this solution was extracted 3 times with 2 ml 0.5 M Tris-HCl buffer, pH 7.5. The ($^{2}H_{8}$)styrene oxide was then extracted from the hexane into 5 ml acetonitrile; this procedure was repeated twice. The solvent was evaporated. The purity and identity of the compound was checked by TLC and HPLC. In addition, quantification of the epoxide was determined through reaction with nicotinamide according to an improved version of the method described by NELIS and SINSHEIMER (1981), and by mass spectrometry.

2.1.3. Preparation of alkylated globins in vitro

Internal standard globin and reference globin were prepared as follows: ($^{2}H_{8}$)styrene oxide or unsubstituted styrene oxide (6 mg) and ^{3}H-labelled styrene oxide (1.7 μCi) were mixed and added to hemolysates from 10 ml samples of human whole blood. The mixtures were incubated for 2 hours at 37 °C and then left at room temperature overnight. Globin was isolated according to MOWRER et al. (1986).

2.2. ANIMAL EXPERIMENTS

2.2.1. Animals and animal care

Female and male NMRI mice, weight 25 to 30 g, and male Sprague-Dawley rats, weight 200 to 280 g, from Eklunds, Vallentuna, Sweden, were used for the experiments. The animals were housed in the animal room at the Arrhenius Laboratories at least one week before onset of the experiments. The room temperature was maintained at 22 °C and the relative humidity at 65%. The animals were fed a standard pellet diet and water without restriction.

2.2.2. Treatment of animals

Styrene or styrene oxide was dissolved in corn oil and administered to the animals by i.p. injection. After 24 hours the animals were anesthetized with diethyl ether and blood was collected. The red blood cells were immediately centrifuged (3,000 g, 10 min) and washed 4 times with 3 volumes of 0.9% NaCl. Red blood cells and blood plasma were then stored at -70 °C.

The following doses, in mmol/kg body weight, were applied to study the dose-response relationship for adduct formation (number and sex of animals given within parenthesis):

rats, styrene:	0 (2 ♂), 0.48 (2 ♂), 1.20 (2 ♂), 2.40 (2 ♂)
rats, styrene oxide:	0 (2 ♂), 0.083 (2 ♂), 0.38 (2 ♂), 1.55 (1 ♂), 1.67 (1 ♂)
mice, styrene:	0 (4 ♀), 0.44 (4 ♀), 2.24 (4 ♀)
	0 (8 ♂), 0.075 (4 ♂), 0.36 (4 ♂), 0.38 (4 ♂), 0.93 (4 ♂), 0.98 (4 ♂), 1.79 (4 ♂), 2.00 (4 ♂)
mice, styrene oxide:	0 (4 ♂), 0.034 (5 ♂), 0.15 (5 ♂), 0.40 (5 ♂), 1.13 (4 ♂)

and the following doses were applied to study adduct stability:

mice, styrene:	0.85 (35 ♀)
mice, styrene oxide:	0.038 (32 ♀)

2.3. QUANTIFICATION OF PROTEIN ADDUCT LEVELS

2.3.1. Characterization of the adduct profile in styrene oxide-treated globin

For this purpose the method of BERGMARK et al. (1990) was used. A sample of 100 mg [^{3}H]styrene oxide-treated human globin (see 2.1.3.) was dissolved in 2 ml of buffer A (see below) and applied to a 26 × 1.6 cm column of CM-Sepharose CL-6B. Immediately following sample application the adsorbed globin chains were eluted by a linear gradient of buffer A to buffer B (total gradient volume = 450 ml). The gradient was maintained using a Pharmacia GM-1 gradient mixer. The flow rate was about 30 ml per hour and fractions of 5 ml were collected. The distribution of protein in the column effluents was determined by absorbance measurement at 280 nm. The radioactivity was determined in 1 ml from each fraction.

The buffers contained 8 M urea and 1 mM dithiothreitol to prevent the oxidation of cysteine residues. The pH was adjusted to 7.2 with phosphoric acid. Buffer A: 0.0175 M disodium hydrogen phosphate, buffer B: 0.035 M disodium hydrogen phosphate.

2.3.2. Preparation and derivatisation of globin samples for determination of N-terminal valine adducts

The preparation and derivatization of globin samples was performed essentially as described by TÖRNQVIST et al. (1986). The conditions for the derivatization were optimized on the basis of exploratory experiments using different concentrations of reagent (PFPITC) and different times of incubation. The following standard procedure was chosen for analysis of globin samples from experimental animals: Samples in the range 20 to 200 mg globin were dissolved in formamide (1.5 ml/ 50 mg) and internal standard globin (50 μg) was added. Pyridine (12 μl/50 mg)* and PFPITC (12 μl/50 mg) were added and the samples were incubated at 45°C for 2 hours with occasional stirring. The samples were extracted three times with 1.5 volumes of diethyl ether and the extracts were evaporated to dryness under nitrogen, redissolved in 2 ml toluene and washed twice with water, 0.1 M disodium carbonate and again with water. This type of extract was used for radioactivity measurements. For GC-MS analysis the toluene was evaporated, and the samples were dissolved in 1 ml methanol : water (60 : 40) and extracted twice with 2 ml hexane. Hexane was evaporated under nitrogen and the samples were dissolved in 50 μl toluene.

2.3.3. Gas chromatographic/mass spectrometric determination of adducts to N-terminal valine in hemoglobin

The derivatized samples were analysed by use of a Finnigan 4500 GC-MS instrument. The gas chromatograph was equipped with an on-column injector (OCI-3; SGE, Ltd., Australia) and a DB-5 fused silica capillary column (30 m, 0.33 mm i.d., 1 μm phase thickness; J. & W. Scientific, Inc., CA) coupled to a deactivated pre-column (1 m, 0.3 mm i.d.) with a glass-lined stainless-steel union (SGE) (TÖRNQVIST et al., 1988). The GC oven was kept at 100°C for 1 min, the temperature was then programmed 15°C/min to 200°C and kept at this temperature for 1 min, then programmed 10°C/min to 300°C and kept at this temperature for 3 min. The mass spectrometer was operated in the negative chemical ionization (NCI) mode, with an ion source temperature of 100°C, and an ionization energy of 100 eV. The ion source pressure was 0.45 torr (60 Pa), and the helium carrier gas pressure was 9 psi (60 kPa).

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* In the original method of TÖRNQVIST et al. (1988) freshly prepared 1 M NaOH is used for neutralization of the globin samples. The use of pyridine for this purpose was considered more convenient. It was shown that neutralization with pyridine or NaOH gave equivalent results in the analysis of hydroxyphenethyl adducts.

The mass spectrometer was either operated in the full scan mode (mass range 50 to 500), or in selected ion monitoring (SIM) mode. In the latter case the mass spectrometer was focused at m/z 424 and 432, the dominating fragments $(M-HF)^-$ for the pentafluorophenylthiohydantoin derivatives of the valine-styrene oxide products and their deuterated variants. Quantification was based on peak areas relative to the internal standard. To check that there was no drift in the ratio of recorded fragments, one of the samples from the calibration curve was reanalysed with each run.

Analysis of styrene oxide was performed using a gas chromatograph (HP 5890), equipped with a mass-selective detector (HP 5970) and a microprocessor workstation (HP 59970 C).

2.3.4. Calibration

A standard curve for hydroxyphenethylvaline-PFPTH was prepared by addition of various amounts of human globin alkylated with unlabelled styrene oxide (reference globin; see section 2.1.3.) and a constant amount (50 µg) of globin alkylated with $(^2H_8)$styrene oxide (internal standard globin) to 25 mg reference globin as a biological matrix. The mixtures were processed up as described above.

The linearity of response in the concentration range of adduct levels was also demonstrated by analyses of mixtures of globin from styrene oxide-exposed and control rats.

2.3.5. Determination of adducts to carboxyl residues in hemoglobin

The method of BERGMARK et al. (1990) was used. Globin samples, ca. 30 mg, from $[^3H]$styrene oxide treated animals were dissolved in water (5 ml). Sulphuric acid was added to give pH 1 and the samples were incubated at 80 °C for 4 hours. Styrene glycol was extracted with ethyl acetate and the radioactivity of the extracts was determined.

2.3.6. Preparation of plasma protein samples

Plasma proteins were precipitated by addition of 1% HCl in acetone. The precipitates were washed with acetone and diethyl ether and were then dissolved in 0.1 M formic acid. The samples were chromatographed on Sephadex G-25 (1.5 × 25 cm) using 0.01 M formic acid as eluent. Fractions of 5 ml were collected. One ml of each fraction was used for radioactivity counting. The protein content was calculated from the absorbance at 280 nm.

2.3.7. Radioactivity measurements

Globin samples, 5 - 10 mg, were dissolved in 1 ml 0.01 M NaOH and counted in 10 ml Picofluor TM_{40} (Packard) (counting time: 9 × 10 min). PFPTH extracts in toluene or extracts of styrene glycol were counted in 3 ml Picofluor TM_{40} (counting time: 10 - 11 × 60 min). Internal standardization was carried out through addition of capsules containing specified amounts of [^{3}H]cholesterol (= 3,300 Bq, 0.09 µCi; LKB Wallac). The measurements were made in a LKB Wallac 1217 Rackbeta liquid scintillation spectrometer.

3. RESULTS

3.1. STRUCTURAL AUTHENTICATION

The chemical structure of the ($^{2}H_{8}$)styrene oxide was confirmed by mass spectrometry. Mass spectra of the commercially available (unsubstituted) and synthetic ($^{2}H_{8}$)styrene oxides are shown in Figures 1a and b. The spectrum of the unsubstituted styrene oxide (Fig. 1a) agrees well with that published by BIDOLI et al. (1980). The corresponding fragmentation pattern of ($^{2}H_{8}$)styrene oxide (Fig. 1b) shows an intensive molecular peak at m/z 128. Loss of 2 mass units (M - ^{2}H) results in the ms peak at m/z 126. Further fragmentation and loss of 18, 30 and 46 mass units results in the peaks at m/z 110, 98 and 82 which correspond to M - $C^{2}H_{3}$, M - $C^{2}HO$ and M - ($C^{2}H$-$C^{2}H_{2}$)O, respectively.

TLC, HPLC and measurements of alkylating activity according to NELIS and SINSHEIMER (1981) showed that the purity of the synthetic ($^{2}H_{8}$)styrene oxide corresponded to that of the commercially available unsubstituted compound.

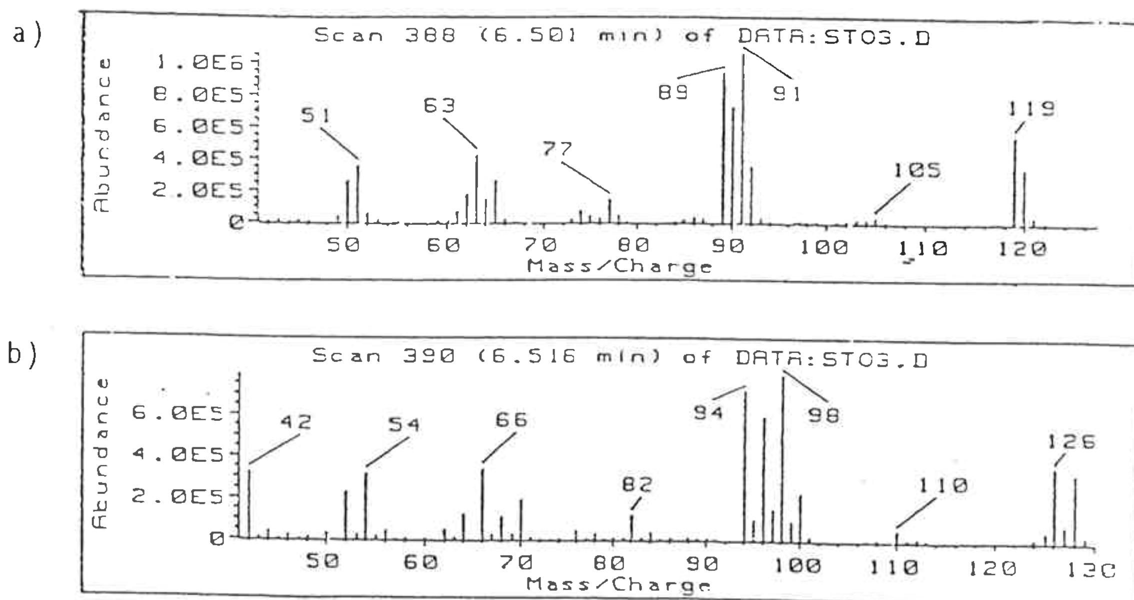

Fig. 1: Mass spectra (EI) of

a) commercially available styrene oxide and

b) synthetic ($^{2}H_{8}$)styrene oxide using an HP 5970 GC-MS

3.2. CHARACTERIZATION OF THE SPECTRUM OF REACTION PRODUCTS (ADDUCTS) IN HEMOGLOBIN

Hydrolysis of globin samples isolated from hemoglobin treated in vitro with radiolabelled styrene 7,8-oxide either in 6 M HCL or by enzyme digestion, gave complicated mixtures of products. An identification of individual components in these mixtures was not carried out within this study.

However, in the investigation of [^{3}H]styrene oxide alkylated human globin by chromatographic analysis (CM-Sepharose CL-6B) it is shown that adducts to valine and carboxyls are quantitatively important (see 2.3.1. and BERGMARK et al., 1990). In the same study it was also demonstrated that this chromatography is useful for globin chain purification and enrichment resulting in improved sensitivity for adduct analysis (biomonitoring).

3.3. STABILITY OF QUANTITATIVELY IMPORTANT ADDUCTS

The stability of hemoglobin and plasma protein adducts was studied after acute exposure of female mice to radiolabelled styrene oxide (0.038 mmol/kg body weight) or styrene (0.85 mmol/kg body weight). The analysis for total binding or specific reaction products was determined at appropriate time intervals during a period corresponding to the life span, t_{er}, of the erythrocytes (40 days in the mouse). Following acute exposures hemoglobin in erythrocytes of all ages are alkylated. Since a constant fraction of the erythrocytes - in the mouse the oldest 1/40 - is eliminated every day, the adduct level decreases linearly with time to zero at time t_{er} if the adduct is chemically stable (SEGERBÄCK et al., 1978).

Most of the adducts to hemoglobin, formed after exposure of mice to styrene oxide, are stable (radioactivity measurements). This is shown in Fig. 2a as a linear decrease of radioactivity, consistent with the normal life span of the erythrocytes. Specific analysis of adducts to N-terminal valine (radioactivity determination of PFPTH derivatives) shows that binding to N-terminal valine is stable (Fig. 2b). The chemical stability of those adducts was also demonstrated by GC/MS analysis of samples obtained from animals exposed to styrene. In contrast carboxyl adducts are eliminated faster than erythrocytes due to hydrolysis of the ester bonds (based on radioactivity determination of adducts released by mild acid hydrolysis; Fig. 2c). It was also found that adducts to carboxyl groups were released from the protein during storage of the acidic precipitate.

Analysis of total binding to plasma proteins in mice treated with radiolabelled styrene oxide showed a logarithmic elimination of radioactivity with an initial

half-life of about 2 days (Fig. 2d). This corresponds to the half-life of serum albumin in the mouse.

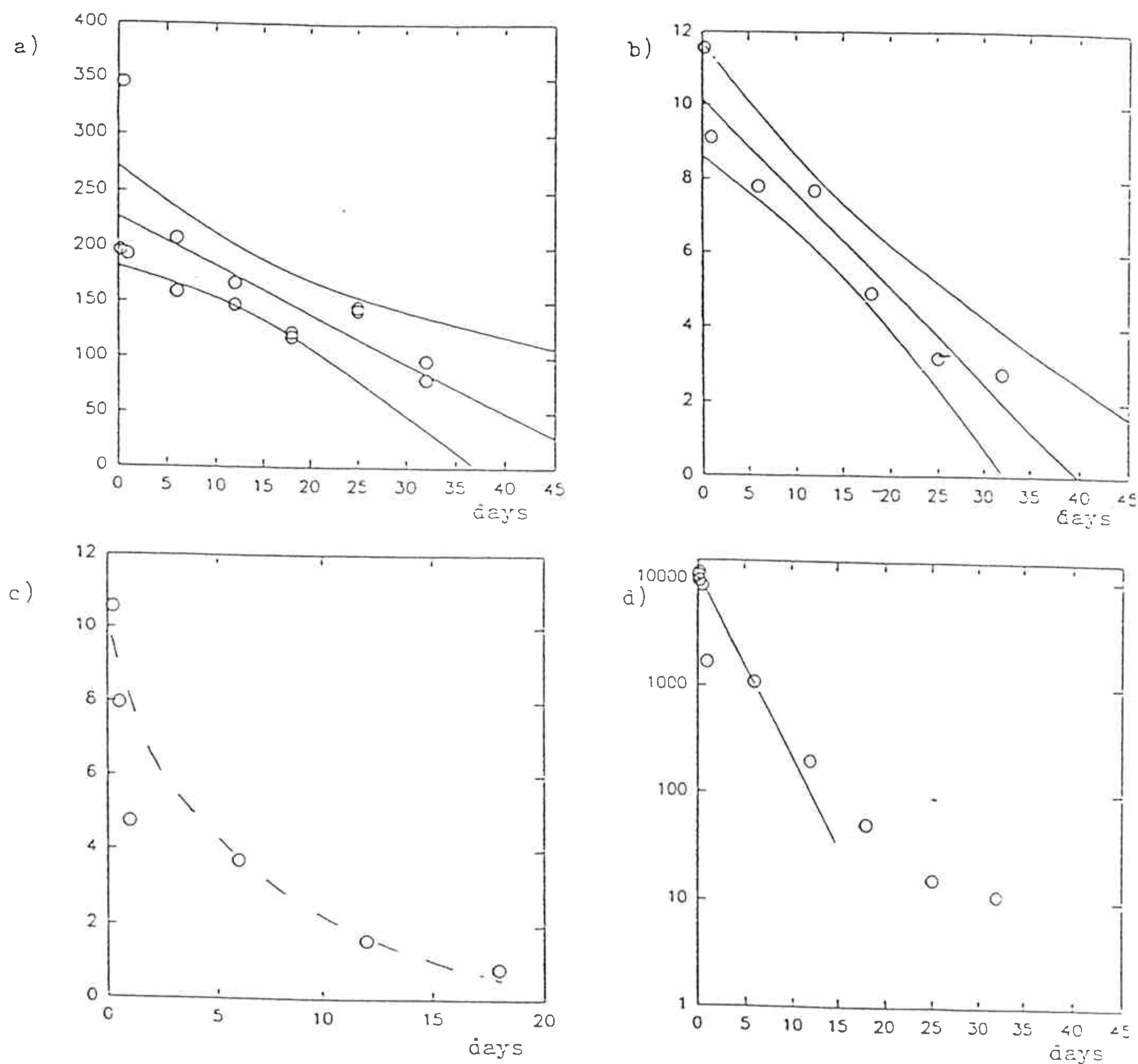

Fig. 2: Binding to blood proteins studied after treatment (at day 0) of mice (female NMRI with [^{3}H]styrene oxide (0.038 mmol/kg body weight; 105 mCi/mmol)

a) Total binding to hemoglobin

b) Binding to N-terminal valine in hemoglobin

c) Binding to carboxyls in hemoglobin

d) Total binding to plasma protein

The Y-axis gives cpm/20 mg protein. Each point represents pooled material from 4 animals. In Figs. 2a) and 2b) the 95% confidence intervals of the regression lines are indicated.

3.4. QUANTITATIVE ANALYSIS OF N-TERMINAL VALINE ADDUCTS

Gas chromatographic analysis of PFPTHs obtained after derivatization of globin alkylated with styrene 7,8-oxide shows two peaks Fig. 3. An identical pattern is seen with the deuterated analogues (data not shown).

Fig. 3: Gas chromatogram of PFPTH derivatives of hydroxyphenylethylvaline in globin from a styrene oxide-treated hemolysate

As shown in Fig. 4 the mass spectra (NCI) obtained from each of these two peaks are similar. The fragmentation patterns indicate that the peaks correspond to the two diastereomers of β-hydroxyphenethylvaline, i.e., the products formed after nucleophilic attack of valine-NH_2 on carbon-8 of styrene 7,8-oxide (see below). However, previous studies of styrene 7,8-oxide, e.g., in reactions with glutathione (HERNANDEZ et al., 1980) or guanosine (HEMMINKI et al., 1986) show that both carbons of the oxirane ring are reactive (see also EHRENBERG and HUSSAIN, 1981). This possibility was not studied further in the present investigation.

The molecular ions of hydroxyphenethylvaline-PFPTH and the corresponding deuterium-substituted derivatives are m/z = 444 and m/z = 452, respectively. By analogy to some studied 2-hydroxyalkylvalines (2-hydroxyethyl- and 2-hydroxypropylvaline) these deriatives lose HF and give M-20 amu, i.e. 424 and 432, respectively, which are the base peaks in these spectra. The fragments 318 and 320, respectively, correspond to additional loss of