ELSEVIER

Journal of Chromatography B, 702 (1997) 77–83

JOURNAL OF CHROMATOGRAPHY B

Ring test for the determination of N-terminal valine adducts of styrene 7,8-oxide with haemoglobin by the modified Edman degradation technique

W. Pauwels[a], P.B. Farmer[b], S. Osterman-Golkar[c], M. Severi[a], R. Cordero[b], E. Bailey[b], H. Veulemans[a,*]

[a]*Laboratory for Occupational Hygiene and Toxicology, Katholieke Universiteit Leuven, Kapucijnenvoer 35/6, 3000 Leuven, Belgium*
[b]*MRC Toxicology Unit, Hodgkin Building, University of Leicester, P.O. Box 138, Lancaster Road, Leicester LE1 9HN, UK*
[c]*Department of Radiobiology, Stockholm University, S-106 91 Stockholm, Sweden*

Received 8 January 1997; received in revised form 1 July 1997; accepted 14 July 1997

Abstract

A ring-test was organised between three laboratories using different versions of the modified Edman degradation technique for the gas chromatographic–mass spectrometric determination of N-terminal valine adducts of styrene 7,8-oxide. The analyses were performed on a sample of human haemoglobin reacted in vitro with styrene 7,8-oxide and on a set of five haemoglobin samples from mice dosed by i.p. injection of styrene. Strong correlations between the haemoglobin adduct determinations of the different laboratories were observed. However, covariance analysis revealed different slopes for the dose–response curves, indicating differences for the calibration of the reference globin or reference peptide. © 1997 Elsevier Science B.V.

Keywords: Edman degradation technique; Haemoglobins; Styrene 7,8-oxide; Valine

1. Introduction

The *N*-alkyl Edman degradation technique is a sensitive method for the determination of adducts of electrophilic agents to the N-terminal valine in haemoglobin [1]. The method has been applied to different groups of chemical agents such as epoxides, methylating agents and aldehydes [2]. The technique has also been used to study the adduct formation by styrene 7,8-oxide, the main metabolite of styrene, in animals [3–5] and men [6,7]. Although the technique generally shows a good reproducibility within the same laboratory, methodological differences between laboratories can possibly lead to important differences in absolute results. Especially methodological differences in the calibration procedure and in the determination of the level of adducts in the standards used, could cause large interlaboratory variations. The present study was undertaken to investigate the degree of interlaboratory agreement in the adduct levels in mice injected with low doses of styrene. For this aim three laboratories, independently doing

*Corresponding author.

PII S0378-4347(97)00380-0

research on styrene adducts, participated in a ring test in which a sample of human haemoglobin, reacted in vitro with styrene oxide, and the same set of haemoglobin samples from mice, dosed by i.p. injection of styrene, were analysed.

2. Experimental

2.1. Animal experiments

Styrene (98% pure stabilised with maximum 15 ppm 4-*tert.*-butylcatechol, UCB, Brussels, Belgium) was mixed with dimethylsulfoxide (analytical grade, Merck, Overijse, Belgium) and corn oil (2:1:9) immediately prior to i.p. injection into young white male NMRI mice (25–30 g) (Charles River, Sulzfeld, Germany). Each of the five different doses of styrene (0–0.86–1.78–3.51–7.43 mmol/kg BW) was administered to six mice. After 3 h the mice were anaesthetised with Nembutal (Sanofi, Brussels, Belgium). The blood was removed by cardiac puncture. After isolation as described by Mowrer et al. [8], the haemoglobin was pooled per dose.

2.2. Preparation of the in vitro sample

To 1 g human haemoglobin (Sigma, St. Louis, MO, USA), dissolved in 7 ml isotonic saline, 0.7 ml styrene 7,8-oxide (97%, Aldrich, Bornem, Belgium) was added. The mixture was stirred at 37°C for 60 h and the reaction was terminated with the addition of 50 ml propanol–HCl (0.05 *M* HCl). After centrifugation the haemoglobin was precipitated from the supernatant by the addition of 50 ml ethyl acetate. The haemoglobin was filtered and washed with 10 ml ethyl acetate (2×) and 10 ml pentane (2×). The globin was dried under a gentle air stream passing over the globin. The sample was diluted 2000 times with blank globin.

2.3. Determination of styrene oxide adducts

2.3.1. Standards and calibration of the reference globin

The standards for the three co-operating labs are compared in Table 1.

Reference globin and I.S. globin for the Louvain laboratory (lab 1) were prepared by the reaction of haemoglobin in saline with styrene oxide and deuterated [$^2H_8$]styrene oxide, respectively [7]. The level of N-terminal valine adduct in the reference globin and the I.S. was determined by acid hydrolysis of the globin, followed by the determination of the amount of alkylated valine as described in Severi et al. [7]. The *N*-(2-hydroxy-2-phenylethyl)valine level in the reference globin was 2.03 nmol/mg globin, whereas the adduct level in the I.S. was 1.73 nmol $^2H_8$-labelled alkylated valine/mg globin.

The Leicester laboratory (lab 2) obtained styrene oxide (97%) from Aldrich (Gillingham, UK). [$^2H_8$]Styrene oxide was prepared according to the procedure of Sepai et al. [9]. Globin preparations (calibration standards) containing adducts of styrene oxide and [$^2H_8$]styrene oxide were prepared by incubating blood with the epoxides according to the procedure of Sepai et al. [9]. The reference globin was made from blood incubated with 1 m*M* styrene oxide and the I.S. was made from blood incubated with 2 m*M* [$^2H_8$]styrene oxide. Only the former was quantitatively analysed for adduct content.

For the determination of the adduct level in the reference globin, aliquots of control globin containing no styrene oxide adducts (50 mg) and of the reference globin, containing unlabelled styrene oxide adducts (5 mg) were hydrolysed in 6 *M* HCl (3 ml containing two drops *n*-octanol) at 110°C for 24 h in vacuo. Aliquots (50 mg) of the $^2H_8$-labelled I.S. globin were hydrolysed similarly. The hydrolysed $^2H_8$-labelled I.S. (corresponding to 1 mg protein) was added to the hydrolysed reference globin samples (aliquots corresponding to 2.5 mg protein) and to a series of calibration line samples. These contained hydrolysed control globin containing no styrene adducts (2.5 mg) plus a range of amounts (0–75 ng) of *N*-(2-hydroxy-2-phenylethyl)valine. All samples were dried and derivatised as follows. To the samples was added redistilled propanol (0.5 ml), 0.5 *M* $KHCO_3$ (0.5 ml) and pentafluorophenyl isothiocyanate (PFPITC) (>97% Fluka, Glossop, UK) (5.0 μl). After mixing, the solution was heated to 45°C for 2 h and then extracted twice with redistilled heptane (3 ml). The solution was taken to dryness in an evacuated centrifuge and the residue redissolved in redistilled toluene (2 ml), washed twice with

Table 1
Description of the standards and the determination of their adduct content for the three co-operating laboratories

	Lab 1	Lab 2	Lab 3
Standard (reference globin/reference peptide)	Globin alkylated in vitro with styrene oxide.	Globin alkylated in vitro with styrene oxide.	Tripeptide, [^{3}H]ValGlyGly ethyl ester, alkylated in vitro with styrene oxide
Calibration of the reference globin	Hydrolysis in 6 *M* HCl of a mixture of the reference globin and a known amount of synthetic *N*-(2-hydroxy-2-[$^2H_5$]phenyl [$^2H_3$]ethyl)valine. Determination of the amount of *N*-(2-hydroxy-2-phenylethyl)valine with previously established calibration curves.	Hydrolysis (separately) in 6 *M* HCl of reference globin, $^2H_8$-labelled I.S. globin and control globin containing no adducts. Analysis of a mixture of hydrolysed $^2H_8$-labelled I.S. globin and hydrolysed reference globin. Determination of *N*-(2-hydroxy-2-phenylethyl)-valine in reference globin using calibration curves established with mixtures of hydrolysed $^2H_8$-labelled I.S. globin, control globin and varying amounts of *N*-(2-hydroxy-2-phenylethyl)valine.	Derivatisation with PFPITC and determination of the radioactivity of the extracted PFPTH derivative.
Adduct level in the reference globin	2.03 nmol/mg globin	15.51 pmol/mg globin	The adducted tripeptide was not isolated, but kept at a stock solution of 680 nmol/ml.
I.S.	Globin alkylated in vitro with [$^2H_8$]styrene oxide.	Globin alkylated in vitro with [$^2H_8$]styrene oxide.	Globin alkylated in vitro with [$^2H_8$]styrene oxide.
Calibration of the I.S.	Hydrolysis in 6 *M* HCl of a mixture of the I.S. and a known amount of synthetic *N*-(2-hydroxy-2-phenylethyl)valine. Determination of the amount *N*-(2-hydroxy-2-[$^2H_5$]phenyl [$^2H_3$]ethyl)valine with previously established calibration curves.	Not determined.	Not determined (see text).
Adduct level in the I.S.	1.73 nmol/mg globin		

water (1 ml) and the toluene layer evaporated again to dryness. The residue was derivatised with acetic anhydride (30 μl) in pyridine (20 μl) at 60°C for 30 min, and the product was dried, reconstituted in acetonitrile, and analysed by GC–MS.

The calibration line was linear ($r^2 = 0.984$) and the amount of *N*-(2-hydroxy-2-phenylethyl)valine in the calibration reference globin was calculated from six determinations as 15.51 pmol/mg globin.

In the Stockholm laboratory (lab 3) a globin, containing adducts of [$^2H_8$]styrene oxide was used as I.S. This standard was prepared by incubating a hemolysate with [$^2H_8$]styrene oxide, mixed with a known amount of [^{3}H]styrene oxide [6]. The adducts of this globin partly degraded during storage (at −20°C for 6 years). The adduct level was not redetermined for this study. An alkylated tripeptide was prepared from [^{3}H]ValGlyGly ethyl ester and styrene oxide as described for the corresponding product of 1,2-epoxybutene [10]. The adduct content was determined by derivatisation with PFPITC and determination of the radioactivity of the PFPTH derivative. A calibration curve was established by adding I.S. and various amounts of the alkylated tripeptide (0.1–50 pmol) to 30-mg samples of control globin. Derivatisation of the samples was carried out as described below. The calibration curve was linear.

2.4. Derivatisation of the samples

The different methods used for the derivatisation of the alkylated haemoglobin are presented in Table 2. Lab 1 and lab 3 followed the method of Törnqvist et al. [1], with only minor modifications, whereas lab 2 used a slightly different method concerning the derivatisation clean-up of the samples. In lab 2, after completion of the initial derivatisation reaction, a SepPak purification step was included. The C_{18} SepPak columns (Millipore UK, Watford, UK) were prewashed with redistilled ethyl acetate, methanol and water (4 ml each). The derivatised samples were diluted with water (3 ml) and washed onto the cartridge columns with more water (4 ml). The columns were washed with redistilled hexane (1 ml) and the product then eluted with ethyl acetate (4 ml). The samples were evaporated to dryness and reacted with acetic anhydride (30 μl) in pyridine (20 μl) at 60°C for 30 min. After this additional derivatisation the samples were dried and redissolved in acetonitrile (30 μl). Lab 3 used extraction with hexane from methanol–water (60:40) for final purification of the samples.

2.5. Gas chromatography–mass spectrometry

The GC–MS conditions used in the three labs are described in Table 3.

2.6. Calibration curves

Standard curves in all labs were prepared through the addition of different amounts of the calibration reference globin (or tripeptide alkylated with styrene oxide) to a constant amount of I.S. All established calibration lines were linear. The samples were

Table 2
Derivatisation and purification of the samples for the three co-operating laboratories

	Lab 1	Lab 2	Lab 3
Sample amount	50 mg globin in 1.5 ml formamide extracted with pentane.	25 mg globin in 2 ml formamide. After addition of I.S., twice extracted with distilled hexane.	50 mg globin in 1.5 ml formamide.
I.S.	10 μl solution containing 1.2 mg globin/ml and 1.73 nmol *N*-(2-hydroxy-2-[$^2H_5$]phenyl[$^2H_3$]ethyl)-valine/mg globin.	30 μl solution containing 0.25 mg globin containing an unknown amount of *N*-(2-hydroxy-2-[$^2H_5$]phenyl-[$^2H_3$]ethyl)valine.	50 μg I.S. containing an unknown amount of *N*-(2-hydroxy-2-[$^2H_5$]phenyl-[$^2H_3$]ethyl)valine.
Reaction medium	30 μl 1 *M* NaOH. 15 μl PFPITC.	10 μl 0.01 *M* NaOH. 10 μl PFPITC.	40 μl 1 *M* NaOH. 10 μl PFPITC.
Reaction conditions	Shaken overnight at room temperature. 90 min at 45°C.	Overnight in rotary mixer at room temperature. 90 min at 45°C.	Shaking for 120 min at 45°C.
Purification	3×2 ml ether and dried under N_2. Dissolved in 2 ml toluene. Washed with water (2×), $NaHCO_3$ (0.1 *M*) and water. Dried under N_2 and redissolved in 50 μl toluene.	C_{18} purification step (see text). Eluted product in ethyl acetate dried in an evacuated centrifuge and dissolved in redistilled 2 ml toluene. Washed with 1 ml water and 1 ml Na_2CO_3 (0.1 *M*). Evaporated to dryness and derivatised with acetic anhydride (30 μl) at 60°C for 30 min. Dried and redissolved in acetonitrile (30 μl).	3×1.5 volume diethyl ether and dried under N_2. Dissolved in 2 ml toluene. Washed with water (2×), Na_2CO_3 (0.1 *M*) and water. Dried under N_2 and redissolved in 1 ml methanol–water (60:40). Extracted twice with 2 ml hexane. Pooled hexane extracts were dried under N_2 and dissolved in 50 μl toluene.

Table 3
GC–MS conditions for the three co-operating laboratories

	Lab 1	Lab 2	Lab 3
Instrument	HP 5890 Series II gas chromatograph. HP5970 quadrupole mass spectrometer.	HP 5980 series II gas chromatograph. VG Trio 1 quadrupole mass spectrometer.	MS/MS Finnigan TSQ-700 instrument.
Column	Alltech DB-5-ms; 30 m×0.32 mm, 0.12-μm phase thickness.	Restek-5 crossbond column; 30 m×0.32 mm, 0.25-μm phase thickness.	Alltech SE-54; 30 m×0.32 mm, 1.0-μm phase thickness.
Injection system	Solid injector; injector temperature 253°C; sample amount: 1–10 μl.	Split/splitless injector; injector temperature 280°C; sample amount: 1 μl.	Varian 1093 SPI (septum-equipped programmable) retention gap: methyl deactivated fused silica (Chrompack; 2.5 m×0.53 mm I.D.). Injector program: at 180°C/min from 70 to 300°C, kept at 300°C for 25 min; sample amount, 2 μl.
Operation mode	Electron impact: source temperature, 270°C; ionising energy, 70 eV; carrier gas, helium (4 psi).	Electron impact: source temperature, 200°C; ionising energy, 70 eV; carrier gas, helium (5 psi).	Negative ion chemical ionisation: reagent gas, methane (5000 mTorr); carrier gas, helium (8 psi, 53 kPa).
Temperature program	Oven program: 5°/min from 150 to 250°C; 10°/min to 300°C.	Oven program: 80°C for 1 min; to 290°C at 30°C/min and kept at 290°C for 6 min.	Oven program: 100°C for 1 min; to 240°C at 20°C/min, then to 320°C at 10°C/min and kept at 320°C for 7 min.
Monitored ions	Adduct: *m/z* 325. I.S.: *m/z* 326.	Adduct: *m/z* 426. I.S.: *m/z* 434.	Adduct: *m/z* 424. I.S.: *m/z* 432.

derivatised and analysed according to the descriptions in Table 2.

2.7. Statistical methods

The statistical analysis was performed with SPSS (Statistical Package for Social Sciences), using SPSS for Windows version 7.0. The Pearson interlaboratory correlation coefficients have been calculated on the adduct levels obtained for all six samples in the three labs. Differences between the labs concerning the dose effect relationships up to 3.51 mmol/kg BW were analysed by the General Linear Model procedure. In this model the lab, the dose and the interaction between both, were included.

3. Results

The N-terminal valine adduct contents of the various globin samples, determined by the three participating laboratories, are presented in Table 4. The differences between the values reported by the laboratories were larger at low adduct levels (coefficient of variation about 40%) than at adduct levels above 100 pmol/g globin (coefficient of variation

Table 4
Levels of *N*-(2-hydroxy-2-phenylethyl)valine determined in the five mice (M1–M5) and one human globin sample reacted with styrene oxide (H1)

Sample no.	Dose styrene (mmol/kg BW)	Lab 1 (pmol/g Hb)	Lab 2 (pmol/g Hb)	Lab 3 (pmol/g Hb)	Mean (coefficient of variation)
M1	0	3.3	n.d.[a]	0.7	1.3 (—)
M2	0.86	49	21	38	36 (39%)
M3	1.78	111	46	85	80 (41%)
M4	3.51	216	142	172	177 (21%)
M5	7.43	1002	646	1021	890 (24%)
H1		906	744	1051	900 (17%)

[a]This value was set to zero for the calculation of the mean.

Table 5
Pearson correlation coefficients between the N-terminal valine adduct levels ($n=6$) determined in the three co-operating laboratories

	Lab 1	Lab 2	Lab 3
Lab 1	1		
Lab 2	0.987	1	
Lab 3	0.995	0.997	1

20%). However, a comparison between laboratories showed very strong interlaboratory correlation coefficients which were highly significant ($P<0.001$). The Pearson interlaboratory correlation coefficients are given in Table 5.

In Fig. 1 the N-terminal valine adduct formation after i.p. administration of styrene is given in function of the dose as an average of the three laboratory results. Due to non-linearity clearly observed for the highest dose, no linear dose–effect correlations could be calculated. The dose–effect relationship was linear up to 3.51 mmol/kg BW for lab 1, whereas the results from the other labs might point to a gradual change to nonlinearity at the doses M3 and M4. Due to the difference in the shape of dose–effect relationship, an improvement of the linear approach was not expected from a transformation of the data.

A covariance analysis, with the dose as a covariable, on the part of the curve up to 3.51 mmol/kg BW revealed no difference between the participating labs, indicating no systematic error (bias) which causes a shift in the line. However, as the interaction between the dose and the lab was highly significant ($p=0.019$), a difference in the slope of the curve between the three participating laboratories was observed. The steepest curve was observed for lab 1, the results of lab 2 showed the lowest slope.

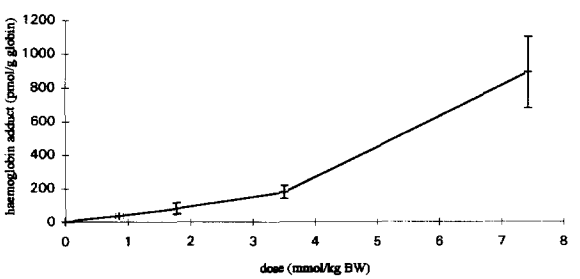

Fig. 1. Dose–response curve for haemoglobin adduct formation (average of the three labs±SD) in the mouse 3 h after i.p. injection with styrene.

4. Discussion

The results between the different labs correlated very well, indicating no major differences in the preparation and the analysis of the samples. The variation in absolute value, obtained in the co-operating laboratories was within a 2.5-fold range. However, the difference in the slope of the dose–response relationship points to a difference in the calibration of the reference globin. Characterisation of the reference globin, or reference peptide, with respect to its adduct content is an essential step in the development of the analytical procedure. Labs 1 and 2 each used a globin, treated in vitro with styrene oxide, as a reference and similar approaches for characterisation of this globin. Lab 1 hydrolysed mixtures of I.S. globin with reference globin or synthetic *N*-(2-hydroxy-2-phenylethyl)valine, respectively, for determination of the adduct level of the reference globin. Lab 2 hydrolysed the I.S. globin and the reference globin separately and mixed the hydrolysate of the I.S. globin with reference globin hydrolysate or with the synthetic adduct before derivatisation and analysis. The quantification of the adduct level of the reference tripeptide used by lab 3 was based on radioactivity determinations. The different results obtained by the three laboratories may in part be related to inaccuracies in the determination of the adduct content of the reference compounds. A clarification of this question would require use of one reference globin (or peptide) available for all laboratories.

To make the application of this method to a general laboratory practice it will be useful to think about simplifying the method at some points. For instance, the shaking of the samples overnight at room temperature (labs 1 and 2) seems to be unnecessary, since lab 3 gets the same results by shaking for 120 min at 45°C.

The non-linearity of the dose–response relationship is in agreement with the results of Osterman-Golkar et al. [4] who observed a faster than linear increase in haemoglobin already at an i.p. injection of 1 mmol styrene/kg BW. The less pronounced

deviation from linearity, clearly observed only between 3.51 and 7.51 mmol/kg BW, is probably due to the short duration of the experiment (3 h). The short duration of the experiment may also explain the lower adduct levels observed in this study as compared for those (6 or 24 h) reported by Osterman-Golkar et al. [4].

Acknowledgments

This project was supported by the Belgian Program for Health Hazards funded by the Services of the Prime Minister; Scientific, Technical and Cultural Affairs. Financial support is also acknowledged from the C.E.C Environmental Research Programme (EV5V-CT920198). Thanks are due to Vlado Zorcec and Hermes Licea Pérez for skilful technical assistance. The Finnigan TSQ-700 MS–MS-instrument was sponsored by the Swedish Council for Planning and Coordination of Research.

References

[1] M. Törnqvist, J. Mowrer, S. Jensen, L. Ehrenberg, Anal. Biochem. 154 (1986) 255.
[2] M. Törnqvist, H. Hindsø Landin, J. Occup. Environ. Med. 37 (1995) 1077.
[3] M. Byfält Nordqvist, A. Löf, S. Osterman-Golkar, S.A.S. Walles, Chem. Biol. Interact. 55 (1985) 63.
[4] S. Osterman-Golkar, A. Christakopoulos, V. Zorcec, K. Svensson, Chem. Biol. Interact. 95 (1995) 79.
[5] W. Pauwels, P. Vodicka, M. Severi, K. Plna, H. Veulemans, K. Hemminki, Carcinogenesis 17(12) (1996) 2673.
[6] A. Christakopoulos, E. Bergmark, V. Zorcec, H. Norppa, J. Mäki-Paakkanen, S. Osterman-Golkar, Scand. J. Work Environ. Health. 19 (1993) 255.
[7] M. Severi, W. Pauwels, P. Van Hummelen, D. Roosels, M. Kirsch-Volders, H. Veulemans, Scand. J. Work Environ. Health. 20 (1994) 451.
[8] J. Mowrer, M. Törnqvist, S. Jensen, L. Ehrenberg, Toxicol. Environ. Chem. 11 (1986) 215.
[9] O. Sepai, D. Anderson, B. Street, I. Bird, P.B. Farmer, E. Bailey, Arch. Toxicol. 67 (1993) 28.
[10] S. Osterman-Golkar, K. Peltonen, T. Anttinen-Klemetti, H. Hindsø Landin, V. Zorcec, M. Sorsa, Mutagenesis 11 (1996) 145.