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Direct determination of styrene-7,8-oxide in blood by gas chromatography with flame ionization detection

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ABSTRACT

A simple capillary gas chromatographic method is described for direct determination of styrene-7,8-oxide (styrene oxide) in blood samples of 1 ml, with a detection limit of 1 ng/ml After the addition of 1-phenylpropylene oxide as internal standard, blood samples were extracted with *n*-hexane, the *n*-hexane phases were concentrated under nitrogen, and up to 25 μl of the resulting solution were injected on-column using a retention gap Separation was carried out on a fused-silica capillary column coupled to a flame ionization detector. Using this method the metabolism of styrene oxide in rat blood was investigated Concentrations of styrene oxide in the blood of rats exposed to styrene at atmospheric concentrations between 20 and 800 ppm for 3 h at steady-state conditions are reported

INTRODUCTION

Styrene-7,8-oxide (styrene oxide) is suggested to be the main first metabolite of styrene in mice, rats and humans (reviewed in ref. 1). Up to now its formation was determined only indirectly by acid conversion into styrene glycol. Styrene oxide was mutagenic in several test systems and tumourigenic in the forestomach of rats after oral application [1]. Styrene oxide is rapidly detoxified by glutathione-S-transferases and epoxide hydrolases present in liver, kidney, and lung [2].

According to Wigaeus *et al.* [3], concentrations of styrene oxide below 10 ng/ml of blood should be expected at workplace exposure concentrations of 20 ppm of styrene (German MAC [1]). The available methods for the determination of styrene oxide in blood are neither sensitive nor specific enough to detect the compound at such low concentrations A method for its direct determination, published by Bidoli *et al.* [4], with gas chromatography–mass spectrometry after extraction of blood with methylene chloride has a detection limit of 10 ng of styrene oxide per ml of blood. A method using gas chromatography (GC) and electron-capture detection after extraction of 4 ml blood with *n*-hexane has a detection limit of 0.5 ng of styrene oxide per ml of blood [3]. Here, styrene oxide was determined indirectly via acid hydrolysis to styrene glycol and subsequent derivatization with pentafluorobenzoyl chloride. However, low amounts of co-

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extracted styrene glycol and other possible *n*-hexane-extractable metabolites, which might be converted into styrene glycol, could falsify the determination.

The purpose of this study was to develop a direct and sensitive GC method that overcomes these shortcomings. It has been used to investigate the metabolism of styrene oxide in rat blood *in vitro*. In addition, we determined the concentrations of styrene oxide in blood after exposure of rats to various concentrations of atmospheric styrene.

EXPERIMENTAL

Chemicals and reagents

(*R*)-Styrene-7,8-oxide (98%), (1*R*,2*R*)-(+)-1-phenylpropylene oxide (98%), styrene (99+%) and diethyl maleate (97%) were from Aldrich (Steinheim, F.R.G.), methanol and *n*-hexane (for residue analysis) and other chemicals (analytical grade) were purchased from Merck (Darmstadt, F.R.G.). Liquemin N 25000 (Heparin-Natrium) was obtained from Hoffmann-La Roche (Grenzach-Wyhlen, F.R.G.). The contamination of styrene by styrene oxide was 30 ppm (w/w) as measured by GC.

Exposure of rats to gaseous styrene

Male Sprague–Dawley rats (220–250 g) (GSF, Neuherberg, F.R.G.) were exposed in closed all-glass chamber systems as described by Filser and Bolt [5]. Styrene was admitted as a liquid or as a vapour into the chamber atmosphere. Initially, the animals were exposed to high concentrations of styrene (1000–3000 ppm) in order to reach steady-state conditions. After *ca.* 4 h the concentration in the gas phase had declined to the desired one of 20, 250, 350, 510 or 800 ppm, respectively. Then the concentration was kept constant (± 10%) for 3 h by repeated administration of styrene into the gas phase of the chamber.

Concentrations of gaseous styrene were measured using a gas chromatograph GC-8A from Shimadzu (Duisburg, F.R.G.) equipped with a 1.5-ml injection loop, a flame ionization detector and a stainless-steel column (2 m) packed with Tenax® GC 60–80 mesh. The temperatures of the oven and of the flame ionization detector were kept constant at 220 and 270 °C, respectively. The flow-rates of nitrogen and hydrogen were 60 ml/min, and that of synthetic air was 600 ml/min.

Collection of blood

Untreated as well as styrene-exposed rats were sacrified using carbon dioxide. After subsequent decapitation, 8–10 ml of blood were collected in ice-cold beakers containing 0.5 ml of heparin solution (Liquemin, diluted 1:10 in bidistilled water). The blood of the exposed rats was instantaneously extracted as described below for the determination of styrene oxide. The blood of untreated rats was immediately used for controls and for *in vitro* experiments.

Extraction of blood

An aliquot of 1 ml blood was transferred to a culture tube (7 ml). Phenylpropene oxide (internal standard, I.S.), 10 ng or 500 ng in 10 μl of methanol, and 1.5 ml of *n*-hexane were added immediately. The culture tube was closed with a PTFE-covered screw-cap then vigorously shaken by hand for 1 min. Subsequently, the phases were separated by centrifugation at 3000 *g* for 2 min and the clear *n*-hexane phase was collected. The extraction with 1.5 ml of *n*-hexane was repeated twice. The combined *n*-hexane phases were concentrated in a calibrated reactivial under a gentle stream of nitrogen to 3, 1 or 0.3 ml, depending on the expected styrene oxide concentration.

Gas chromatography

The GC determination of styrene oxide together with the I.S. was done using a 3400 gas chromatograph from Varian (Darmstadt, F.R.G.) equipped with an on-column injector and a flame ionization detector. Chromatographic runs were integrated using an HP 3396A integrator from Hewlett-Packard (Bad Homburg, F.R.G.) or a PC integration pack from Kontron (Eching, F.R.G.)

Amounts of up to 25 μl of the *n*-hexane extract were injected on-column into the gas chromatograph according to Grob, Jr. *et al.* [6]. Injections were carried out using a 25-μl syringe 25A-RN-GP with an on-column needle SS170/VAA, both from SGE (Weiterstadt, F.R.G.). Samples were separated on a 10 m × 0.32 mm I.D. fused-silica retention gap coupled by a press-fit connector to a 25 m × 0.25 mm I.D. fused-silica column DB 17 (0.25-μm film of 50% phenylsilicone and 50% methylsilicone), all obtained from Carlo Erba (Hofheim, F R.G.).

After injection at 70°C the injector was heated to 200°C at 20°C/min and immediately cooled to 70°C. The oven temperature was kept at 70°C for 2.5 min. Then it was increased to 90°C at 1.5°C/min and to 220°C at 8°C/min where it was kept for 10 min before it was cooled to 70°C.

The carrier gas was helium at a flow-rate of 1.5 ml/min, cleaned of residual oxygen and moisture by the OMI-1 tube from Supelchem (Sulzbach/Taunus, F.R.G.) The temperature of the flame ionization detector was set to 300°C. The detector make-up gas was nitrogen, at a flow-rate of 30 ml/min.

When styrene oxide was determined at concentrations below 10 ng/ml of blood, the column was cleaned after *ca.* 100 injections by inversely flushing it repeatedly with liquid methanol and *n*-hexane at room temperature.

Quantification

Peaks of styrene oxide and of the I.S. in gas chromatograms were identified by their relative retention times, which were based on the beginning of the solvent peak. Solutions of styrene oxide, 1–100 ng per 0.3 ml of *n*-hexane and 1–100 ng/ml of blood, were prepared in the presence of 10 ng of I.S. Extracts from blood were concentrated to 0.3 ml. The standard curve was constructed from ratios of the peak area of styrene oxide to that of the I.S., plotted against the respective

concentrations of styrene oxide. To evaluate the linearity of the method over a large concentration range, solutions of styrene oxide in *n*-hexane and in blood from 10 ng/ml to 100 μg/ml in the presence of the I.S. at 500 ng/ml were prepared. Extracts from blood were concentrated to 3 ml.

The recovery of styrene oxide (1–100 ng per 0.3 ml) and of the I.S (10 ng per 0.3 ml) extracted from blood was calculated by relating the peak areas of the standard curve in blood to the respective peak areas of the means of the calibration curve prepared in *n*-hexane.

Precision

The reproducibility of the GC method was determined by injecting ten times 25 μl of a 0.3-ml *n*-hexane solution containing 10 ng of styrene oxide and 10 ng of the I.S.

The reproducibility of the complete method was determined by extracting and analysing eight samples of 1 ml of blood to which 20 ng of styrene oxide and 10 ng of the I.S. had been added.

Stability of styrene oxide and the I.S. in n-hexane

One sample of a solution of 100 ng of styrene oxide together with 100 ng of the I.S. in 1 ml of *n*-hexane were kept at room temperature, and two others were stored at -25°C. Each sample was measured at 0, 24 and 48 h.

Metabolism of styrene oxide in blood

Styrene oxide in a maximum of 130 μl of methanol was added to 13 ml of blood at 37°C. The concentrations yielded were 1, 10 and 100 μg of styrene oxide per ml of blood. Incubation was performed in 13-ml vials, which were closed by a PTFE-covered septum. The blood was gently stirred for 0.5 min, and samples of 0.5 ml were successively taken over time periods up to 5 h. The I.S., 500 ng in 10 μl of methanol, was immediately added to the sample. Then the sample was extracted with *n*-hexane as described above.

Some experiments were carried out with blood samples that had been preincubated for 30 min at 37°C with diethyl maleate at a final concentration of 10 m*M* in order to deplete GSH [7].

Half-lives were calculated from the slopes of the concentration–time curves obtained in semi-logarithmic plots by linear regression analysis.

RESULTS AND DISCUSSION

Selectivity and specificity

The GC method developed is based on a special on-column injection technique for large sample volumes [6]. Fig. 1a shows a chromatogram obtained after the injection of 25 μl of a solution containing 10 ng of styrene oxide plus 10 ng of the I.S. in 0.3 ml of *n*-hexane. Fig. 1b and c show chromatograms that were obtained

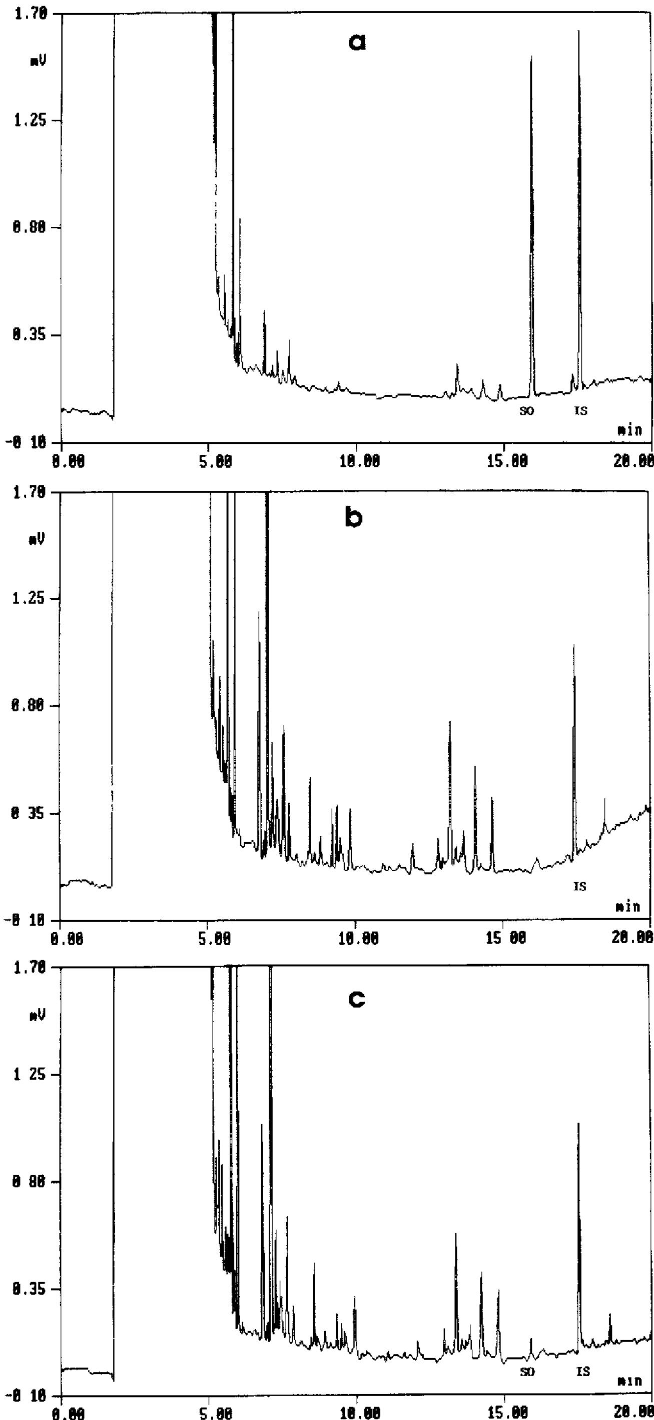

Fig 1 Gas chromatograms of styrene oxide (SO) and the internal standard (IS) after injection of 25 μl. (a) Solution of 33 ng of styrene oxide and 33 ng of internal standard per ml of pure *n*-hexane (b) *n*-Hexane extract of 0 3 ml of 1 ml of rat blood to which 10 ng of internal standard had been added (c) *n*-Hexane extract of 0.3 ml of 1 ml of rat blood to which 1 ng of styrene oxide and 10 ng of internal standard had been added

with extracts from blood samples of 1 ml to which 10 ng of the I.S. or 10 ng of the I.S. plus 1 ng of styrene oxide had been added. The retention times related to the beginning of the solvent peak were 14 min for styrene oxide and 15.6 min for the I.S. Comparison of the chromatograms reveals that there is no interfering signal at the retention time of styrene oxide. Owing to the cold injection no decomposition of styrene oxide took place that could occur at the high temperature of a split–splitless injector.

Precision, recovery and detection limit

Styrene oxide and the I.S. were extracted from blood with the non-polar solvent *n*-hexane in order to minimize possible interferences caused by polar compounds during the separation. The reproducibility of the GC method itself resulted in a coefficient of variation (C.V.) of 7%. The reproducibility of the complete method was only slightly higher, with a C.V. of 10% (Table I).

The calibration curve obtained with 1–100 ng of styrene oxide per ml blood in the presence of 10 ng of the I.S. per ml of blood was a straight line through the origin. The linear regression analysis performed on the ratios of the peak area of styrene oxide to that of the I.S. *versus* the styrene oxide concentrations gave $y = 0.102x - 0.01$ and $r = 0.9997$. A similar correlation coefficient of 0.9999 resulted from the calibration curve obtained from the analysis of 10 ng–100 μg of styrene oxide per ml blood in the presence of 0.5 μg of the I.S. per ml. The C.V. at each styrene oxide concentration was less than 15% ($n = 3$).

Extraction recoveries of styrene oxide at concentrations between 1 and 100 ng/ml of blood are given in Table II. The mean value of all concentrations was 75%. The I.S. had a mean recovery of 72% at 10 ng/ml of blood. Wigaeus *et al.* [3], who also used *n*-hexane, did not provide information. Bidoli *et al.* [4] published a recovery of 93% after extraction of mouse blood with the more polar methylene chloride.

Since large volumes can be injected on-column, a high sensitivity can be

TABLE I

REPRODUCIBILITY OF THE MEASUREMENT OF STYRENE OXIDE AND INTERNAL STANDARD IN PURE *n*-HEXANE AND RAT BLOOD

Compound	Concentration (ng/ml)	*n*	SO/I.S. (area/area)	Coefficient of variation of SO/I.S. (%)
n-Hexane				
Styrene oxide	30	10	1.01	7
Internal standard	30	10		
Rat blood				
Styrene oxide	20	8	2.08	10.1
Internal standard	10	8		

TABLE II

RECOVERIES OF STYRENE OXIDE AND THE INTERNAL STANDARD FROM RAT BLOOD

Calculation was done by relating the peak areas obtained from *n*-hexane extracts from blood to the mean peak areas of the respective solutions of styrene oxide and internal standard in pure *n*-hexane

Concentration in blood (ng/ml)	Recovery (mean ± S.D) (%)	*n*
Styrene oxide		
1	85 ± 8 2	3
5	76 ± 7 9	3
10	71 ± 6 3	3
50	78 ± 4.4	3
100	67 ± 2 3	3
Mean value	75 ± 9	5
Internal standard		
10	72 ± 8	8

achieved with this technique. At an injection volume of 25 μl, the method presented revealed a detection limit of 1 ng of styrene oxide per ml of blood at a signal-to-noise ratio of 6:1.

Stability of styrene oxide

Proper handling of *n*-hexane extracts and blood samples containing styrene oxide requires knowledge of its stability in both liquids.

TABLE III

STABILITY OF STYRENE OXIDE AND THE INTERNAL STANDARD IN PURE *n*-HEXANE AT −25°C AND ROOM TEMPERATURE

Time (h)	Peak area at 100 ng/ml		SO/I S.
	SO	I S	
−25°C			
0	223 400	190 400	1 173
24	222 600	187 800	1 185
48	222 100	189 700	1.171
0	226 500	197 100	1.149
24	226 900	198 700	1 142
48	226 100	196 900	1 148
Room temperature			
0	218 000	220 379	0 989
24	217 900	222 300	0 980
48	216 300	219 400	0.986

If styrene oxide and the I.S. were stored in *n*-hexane their concentrations did not change within two days, either at −25°C or at room temperature (Table III).

When styrene oxide was incubated in rat blood at 37°C, its concentration fell rapidly giving concentration–time courses with slopes which increased with decreasing concentrations (Fig. 2). This cannot be explained by the loss of styrene oxide into the gas phase of the vessels owing to the subsequent removal of blood samples. Otherwise, the concentration–time plots obtained with an initial concentration of 1 μg/ml (n = 4) would also be curved. However, such concentration–time plots are characteristic for saturation kinetics. In the range between 100 and 10 μg of styrene oxide per ml of blood the half-life was 32 ± 3.5 min (mean ± S.D.; n = 4) and in the range between 10 and 1 μg/ml it was 24.5 ± 3.1 min (mean ± S.D., n = 8). At concentrations below 1 μg/ml the half-life was 18.2 ± 3.5 min (mean ± S.D.; n = 11). One blood specimen showed a half-life of only 10 min in the range between 10 and 1 μg/ml (data not given). The degradation seems to be dependent on a saturable glutathione-S-transferase activity in red blood cells [8]. This assumption is supported by the much higher stability of styrene oxide that was observed in two experiments after preincubation of the blood for 30 min with 10 m*M* diethyl maleate to deplete GSH [7]. The resulting concentration–time plots courses are also shown in Fig. 2: the mean half-life of styrene oxide was 310 min.

Styrene oxide in blood after exposure to gaseous styrene

Styrene has been shown to be metabolized by microsomal monooxygenases of various organs [9–11]. However, the assumed main metabolite, styrene oxide, was detected only indirectly by measuring the formation of styrene glycol. In the present study, we determined the occurrence of styrene oxide itself in the blood of

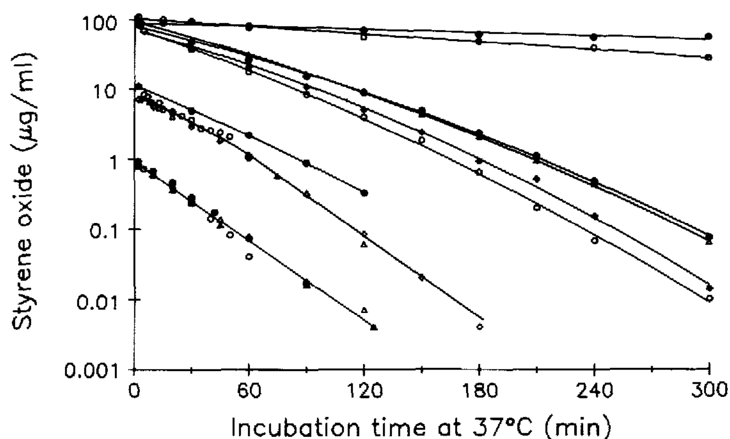

Fig. 2. Stability of styrene oxide in rat blood at initial concentrations of 1, 10 and 100 μg/ml. Blood samples of 13 ml were incubated in closed vials (13 ml) at 37°C. Each set of symbols represents a single experiment The two upper curves were obtained with blood preincubated over 30 min with 10 m*M* of diethyl maleate at 37°C. Data were fitted visually.

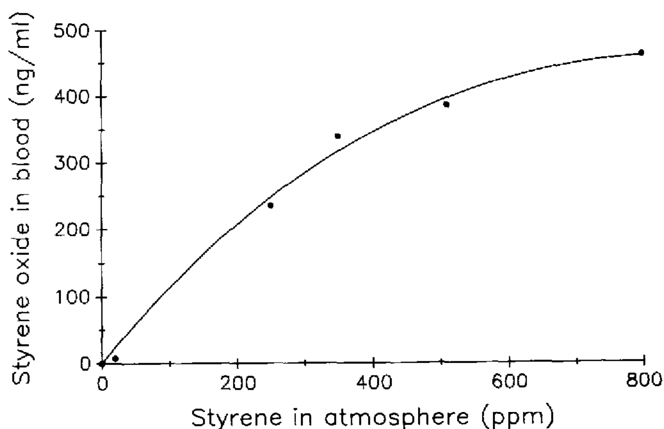

Fig 3 Styrene oxide concentrations in blood of rats exposed to atmospheric styrene of 20, 250, 350, 510 and 800 ppm at steady state for 3 h. Each point represents the mean of three measurements of styrene oxide in the blood of one rat Data were fitted visually

rats exposed to various concentrations of atmospheric styrene (Fig. 3). A styrene oxide concentration of 7.7 ng/ml of blood was found at the lowest styrene concentration of 20 ppm. The observed non-linear relationship between the concentrations of the two substances can be attributed to the saturation of the metabolism of styrene [12,13].

CONCLUSIONS

We have developed a method for the determination of styrene oxide in rat blood that does not require complex and expensive equipment. It is sensitive and precise enough to detect styrene oxide after exposure of rats to atmospheric styrene below the current German MAC value.

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