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Determination of styrene-7,8-oxide in whole rat blood by gas chromatography–mass spectrometry

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ABSTRACT

A rapid and selective method for the determination of styrene-7,8-oxide (SO) in whole blood has been developed. Blood samples as small as 0.1 ml are extracted once with benzene containing phenyl propylene oxide as an internal standard. The extracts are analyzed by gas chromatography–mass spectrometry using an automated cold on-column injection system to avoid thermal rearrangement of SO to phenylacetaldehyde. The overall mean recovery ($\pm$ 2 σ) of SO from fortified blood samples was 92 $\pm$ 21% and the detection limit was 10 ng/g. Results of experiments examining the half-life of SO in whole rat blood are presented. The method was also used to analyze sequential blood samples from rats administered SO orally.

INTRODUCTION

Styrene-7,8-oxide (SO) is a labile metabolite of styrene [1] and is generally considered to be responsible for much of the toxicity associated with styrene [2]. Styrene is oxidized to SO via microsomal cytochrome P-450-dependent enzymes. SO in turn undergoes spontaneous and enzymatic hydrolysis to phenylethylene glycol (PG), and is also conjugated with glutathione. PG is then either further oxidized or conjugated with glucuronic acid prior to excretion. Due to the rapid rate at which SO is hydrolyzed and conjugated, levels of SO in the blood during and following an exposure to styrene are expected to be very low. Nevertheless, Wigaeus *et al.* [3] reported finding an average of 6 ng SO per ml of blood drawn from a human volunteer after a 2-h inhalation exposure to 70 ppm styrene. However, this report requires confirmation because the method used to measure SO involved indirect determinations.

A rapid, selective method for the determination of SO in rat blood was needed to support a planned kinetic study. This kinetic study required the analysis of multiple, small-volume ($\sim$ 0.1 ml) blood specimens taken from individual rodents. This sampling regimen allows the kinetics to be defined in individual animals and thus minimize the number of animals needed. In addition, the analytical

method needed selectivity, particularly in regard to known or potential metabolites of styrene such as PG or phenylacetaldehyde. Previously reported methods either did not have the desired 10 ng/g detection limit, required much larger volumes of blood, or involved procedures which could possibly compromise the specificity of the measurement [3–5]. Notably, the previous procedure called for liquid–liquid extraction of SO, from samples containing 100-fold more PG than SO, followed by conversion of SO to PG prior to derivatization. Thus even minimal carryover of PG could yield erroneous results.

The method described here is rapid, requires only small sample volumes, and monitors the intact SO molecule.

EXPERIMENTAL

Materials

(*R*/*S*)-Styrene-7,8-oxide (98%, Lot No. 03130KM), (±)-1-phenyl-1,2-ethanediol (97%, Lot No. 08310LP and (1*R*,2*R*)-(+)-phenylpropylene oxide (PPO, 99%, Lot No. 50906HM) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Benzene was procured from Burdick and Jackson (Muskegon, MI, U.S.A.). Acetonitrile and anhydrous sodium sulfate were acquired from Fisher Scientific (Midland, MI, U.S.A.). Whole blood was obtained by cardiac puncture from male Fischer 344 rats (Charles River Breeding Laboratories, Kingston, NY, U.S.A.) anesthetized with carbon dioxide. Blood was collected using heparinized Vacutainers® (Beckton-Dickinson, Rutherford, NJ, U.S.A.).

Preparation of solutions

A PPO (internal standard) stock solution was prepared by adding PPO to benzene and diluting to yield 0.3 mg/ml. The extraction solution used both for the extraction of blood samples and for preparation of analytical standards was made by diluting the above stock solution with benzene to yield 0.3 μg/ml PPO. Analytical standards containing 1000–0.01 μg/ml SO and 0.3 μg/ml PPO were generated by adding 7.5–25 μl of SO to 10 to 50 ml of extraction solution and also serially diluting some of those standards with additional extraction solution to achieve the lowest concentrations.

Blood samples used in validation studies were fortified with SO using acetonitrile as the solvent. Spiking solutions were prepared by adding SO directly to acetonitrile and diluting to appropriate volumes. The concentrations of the spiking solutions were chosen such that the desired amount of SO could be delivered in 2–20 μl of solvent per g of blood. Similar acetonitrile solutions containing PPO were also prepared.

Sample preparation

Whole blood (0.1–0.3 g) was added to clear glass vials (1 or 4 ml) containing 100–300 mg anhydrous sodium sulfate and 0.1–1.5 ml extraction solvent. The

vials were capped immediately using Teflon-lined septa caps and the samples were agitated vigorously for approximately 15 s using a vortex mixer. The samples were then centrifuged for approximately 5 min to precipitate lysed red blood cells into a solid pellet. The supernatant liquid was then transferred to a clear glass autosampler vial and capped with a Teflon-lined crimp-top cap. Low-volume conical autosampler vials were used for sample volumes less than 0.5 ml.

Gas chromatography–mass spectrometry

A Hewlett-Packard 5970B mass-selective detector (MSD, Palo Alto, CA, U.S.A.) was used for this determination. The MSD was equipped with an HP 7673 autosampler, cold on-column injector, and an automated reduced-pressure interface device [6]. A 4 m × 0.53 mm deactivated retention gap (Restec, Bellefonte, PA, U.S.A.) connected with a glass butt connector to a 10 m × 0.25 mm DB-5 capillary column (J&W Scientific, Folsom, CA, U.S.A.) was used for the chromatographic separation. Samples (1 μl) were injected automatically. The column oven was held at 35°C for 0.25 min, programmed at 40°C/min to 220°C, and held there for 0.63 min. The interface temperature was 280°C. Helium was used as the carrier gas at a head pressure of 0.69 bar. Electron-impact ionization (70 eV) was used. The fragment ion at m/z 89 was monitored for both SO and PPO using a dwell time of 100 ms. An electron multiplier voltage of 1800–2200 V was used. The MSD was first-tuned automatically and then manually using m/z 69, 131, and 100 as reference peaks. The ion offset voltage was then lowered until the peak widths were near unity.

Validation procedures

The linearity of the response was evaluated over a range of concentrations from 0.01 to 10 μg/ml SO in analytical standard solutions containing 0.3 μg/ml PPO. Recovery and precision evaluations were carried out by fortifying 0.1–0.3 g of whole blood with 2 μl SO spiking solution to yield concentrations of 0.01–10 μg/g (one sample set also included PG at 120 times the SO concentration). Fortified blood samples were shanken by hand for 5 s to facilitate mixing and then extracted immediately as described above.

Because the time between extraction and analysis will vary for samples in large batches, the recovery of SO was evaluated over a six-day period in which the supernatant extract was stored at room temperature over the red blood cell precipitate in the original extraction vial. In addition, the stability of standard solutions was evaluated after 74 days of storage at room temperature.

During actual kinetic studies, blood samples are withdrawn from rats using indwelling Silastic® cannulae, thus, the effect of these cannulae on SO recovery was examined. Blood SO concentrations (1 μg/g) were measured before and after passage through a 6-in. Silastic® cannula. Because corn oil is used as a dosing vehicle in studies of SO administered orally, the stability of SO in corn oil (250 mg/g) was examined when stored at room temperature on the lab bench for 39 days in a clear glass vessel.

Stability in whole blood

The half-life of SO in rat blood (10 μg/g) was determined by sequentially sampling from sealed vessels maintained at room temperature (22.5°C) and at the body temperature of the rat (37°C). In a second experiment kinetics were determined at two concentrations (100 and 10 μg/g). At the 10 μg/g level in this latter experiment a second equal addition of SO was made to the same vessel at ~ 100 min. The blood used in these experiments had been taken from non-fasted rats early in the morning. Experiments were initiated approximately 2–3 h after procuring the blood.

In all blood stability experiments SO was added as 10 μl of acetonitrile solution to approximately 10 g of blood in clear glass vials. In all experiments, a small Teflon-coated magnetic stirring bar was placed in each vial and the blood added, leaving only a small (<0.1 ml) headspace. The vials were then capped with Mininert® valve caps. Mixing was effected manually by inverting the vials end to end for 30 s. Blood samples were then removed periodically and extracted as above.

Styrene oxide oral administration to rats

Male Fischer 344 rats weighing approximately 200 g were anesthetized with methoxyflurane and a Silastic cannula implanted in their right jugular vein [7]. Two days post-surgery, groups of four rats were given a single oral dose of either 275 or 550 mg/kg of SO dissolved in corn oil. The animals were then placed in a restrainer (Centrap® cage, from Fischer Scientific, Pittsburgh, PA, U.S.A.) and sequential blood specimens of 0.1–0.2 ml were collected via the jugular cannula just prior to dosing and at 5, 15, 30, 45, 60, 90, 120, 150, 180, 240, and 300 min post-dosing. The blood specimens were immediately weighed, extracted with benzene containing PPO as an internal standard, and analyzed for SO as described above.

RESULTS AND DISCUSSION

When evaluating the direct determination of SO by GC–MS, it was found that a significant portion of the injected SO was undergoing thermal rearrangement to form phenylacetaldehyde during the conventional splitless injection procedure. Phenylacetaldehyde formed during injection appeared as a tailing peak that eluted prior to SO. The relative amount of phenylacetaldehyde formed increased as the concentration of SO injected decreased. The use of the Hewlett-Packard automated cold on-column injection system eliminated this thermal rearrangement phenomenon.

Benzene was used as the extraction solvent is this work because it provided a high extraction efficiency, yielded a clean supernatant extraction layer, and exhibited good chromatographic qualities for cold on-column injection. Potential for exposure to benzene is minimized by using small aliquots, filling all extraction

vials prior to the experiments so blood samples could be added to the solvent in the capped vials, and carrying out all transfers of benzene solvent in an appropriate fume hood.

The electron-impact ionization mass spectra obtained under these GC–MS conditions are shown in Fig. 1. Both SO and PPO exhibit abundant molecular ions at m/z 120 and 134, respectively. The fragmentation pathway of aromatic epoxides related in structure to SO and PPO have been proposed in studies using high-resolution MS and stable isotope labeling [8]. Loss of the benzylic hydrogen gives rise to abundant M − 1 ions. Transannular bond rupture without hydrogen rearrangement yields ions at m/z 90 and loss of an additional hydrogen atom yields ions at m/z 89. These two ions are ring expansion products. Fragmentation to form these ring expansion products at m/z 89 and 90 is reported to be absent at 12 eV. In this work m/z 89 was chosen to monitor both SO and PPO because it is a relatively unique ion and because the other predominant ions in the spectra are common to a multitude of other chemicals. While chemical ionization MS could potentially provide greater sensitivity for SO, electron-impact ionization was chosen because it yielded the sensitivity needed for this work and often provides more reliable operation.

Typical ion chromatograms obtained by this method are shown in Fig. 2. Under these conditions, SO elutes at about 5 min with PPO eluting about 10 s

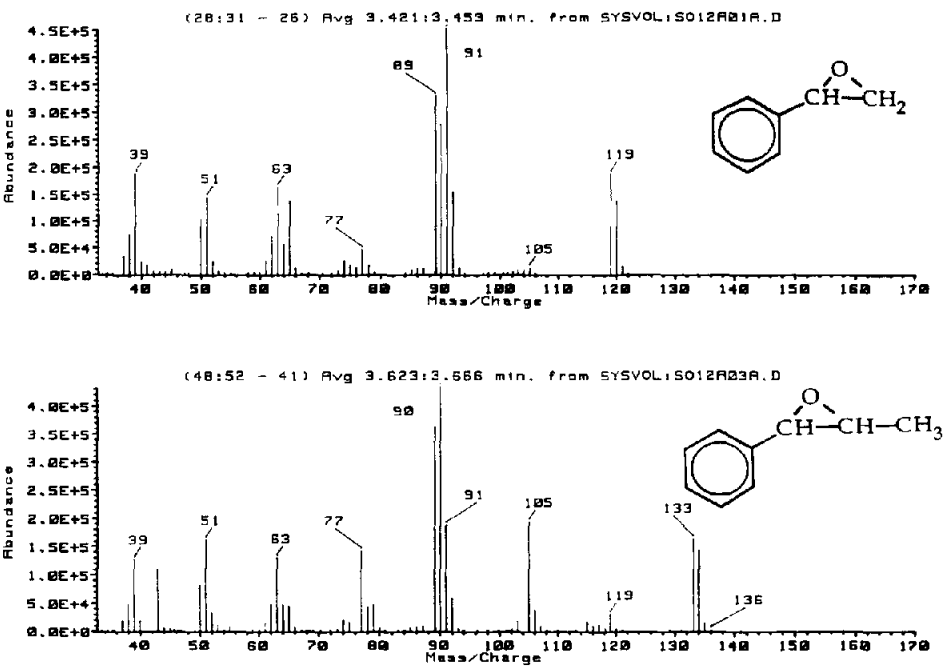

Fig. 1. Electron-impact mass spectra of styrene-7,8-oxide and 1-phenyl-1,2-propylene oxide.

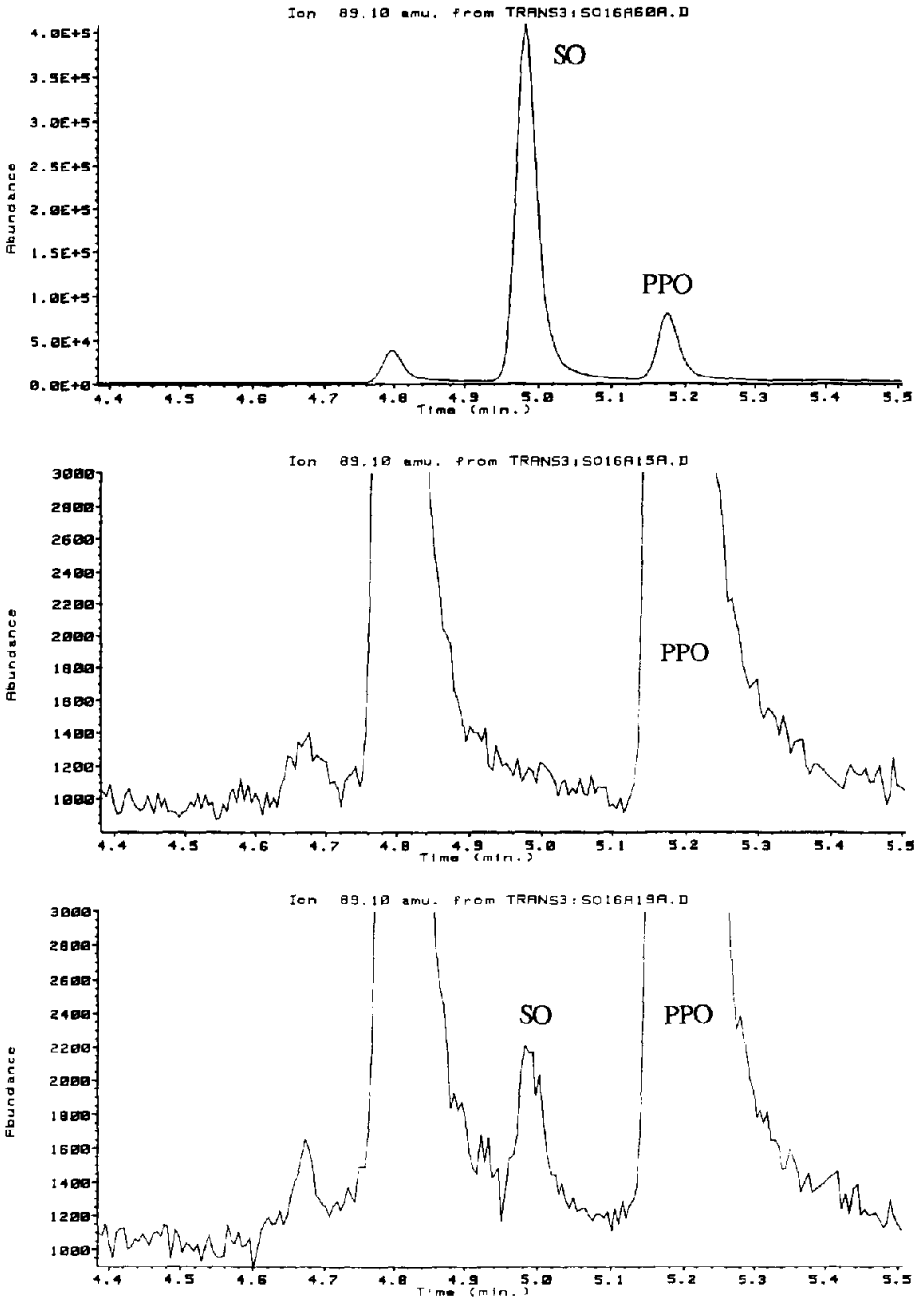

Fig. 2. Typical chromatograms for the determination of styrene-7,8-oxide in whole rat blood. The above ion chromatograms (*m/z* 89) show (top to bottom) extracts of a blood sample containing 1.0 μg/g SO, a control blood sample, and a blood sample containing 0.01 μg/g SO. The internal standard, PPO, is present at 0.3 μg/ml in all samples. The peak eluting at ~4.8 min is *trans*-β-methylstyrene, an internal standard to be used for styrene determinations.

later. The peak eluting at about 4.8 min in these chromatograms is *trans*-β-methylstyrene, an internal standard to be used in the determination of styrene. No interferences have been observed under these conditions between SO and PPO or from the sample matrix. The detection limit for this method defined as three times the peak-to-peak background noise is 0.01 μg/g.

Table I lists typical response factors obtained for analytical standards. The relative response for PPO and SO becomes non-linear above 1 μg/ml. This non-linearity may be due in part to changes in the electronic peak integration parameters as the SO peak increases significantly higher than the PPO peak, but could also be due to overloading of the capillary column. Samples known to contain greater than 1 μg/ml SO can be diluted into the linear range. Alternatively, additional standards can be analyzed and the response factors extrapolated between bracketing standards.

The stability of standard solutions containing SO and PPO in benzene was evaluated. The mean response factor obtained from standards stored for 74 days on the lab bench at room temperature was unchanged from that obtained on day 1 (−3% relative).

Table II lists the recovery of SO from fortified blood samples carried out on two different days. The overall mean recovery for 34 samples was 92%. The overall precision (95% confidence interval) was ± 21%. These data indicate that the rapid sample preparation scheme in this method allows accurate determination of actual blood SO concentrations. It should be noted that in recovery experiment 1, PG was included in the samples at concentrations 120 times greater than SO. These data confirm that PG does not interfere with this SO determination.

The stability of SO in several different media was evaluated. The mean recovery (± 1 S.D.) of SO from a 250 mg/g corn oil dosing solution was 94 ± 2% after 39 days. Five benzene-spiked extracts of whole blood reanalyzed after storage at room temperature in clear glass vials for six days in contact with red blood cell precipitates yielded results 99 ± 7% (mean ± 1 S.D.) of the original analysis. These data indicate SO concentrations are stabilized by the extraction process.

TABLE I

LINEARITY OF RELATIVE RESPONSE

Concentration (μg/ml)	Response factor (mean ± R.S.D.)	*n*
0.02	1.31 ± 1	2
0.06	1.38 ± 13	2
0.11	1.37	1
0.32	1.38 ± 3	7
0.63	1.42 ± 5	3
1.05	1.48 ± 6	3
10.5	2.74 ± 7	2

TABLE II

RECOVERY OF STYRENE-7,8-OXIDE FROM WHOLE RAT BLOOD

Concentration (μg/g)	Recovery (%)	n
Experiment 1		
0.01	96 ± 3	2
0.05	93 ± 7	4
0.10	93 + 9	2
0.50	92 ± 1	2
1.00	84 ± 4	2
10	86 ± 8	2
Mean ± S.D.	91 ± 6 (n = 14)	
Precision	13	
Experiment 2		
0.02	100 ± 21	6
0.03	92 + 8	2
0.06	95 ± 8	2
0.10	89 ± 2	4
0.30	82 ± 0.1	2
0.60	88 ± 9	2
1.00	91	1
10.0	94	1
Mean ± S.D.	92 ± 13 (n = 20)	
Precision	26	
Overall mean	92	
Overall S.D.	11	
Precision	21	

When whole blood containing 1 μg/g SO was passed through Silastic tubing, typical of that used as indwelling cannulae, the mean recovery (± 1 S.D., n = 3) was 91 ± 4%. In actual kinetic studies results for blood samples collected via indwelling cannulae are corrected for this recovery factor as well.

Prior to initiating a kinetic experiment, some knowledge of the stability of SO in whole blood was needed in order to design appropriate sample handling procedures. Two experiments were carried out (Fig. 3). In the first experiment the half-life of SO added to whole blood at an initial concentration of 10 μg/g was determined by sequentially sampling from sealed vessels (duplicates at each temperature) maintained at room temperature (22.5°C) or at the body temperature of the rat (37°C). The half-life of SO in whole blood at 22.5°C was found to be 84 min and the half-life at 37°C was 26 min.

In a second experiment, all vessels were maintained at 37°C and two initial concentrations, 10 and 100 μg/g, were used. In addition, when the 10 μg/g samples reached about 1 μg/g after approximately 100 min, a second 10 μg/g SO

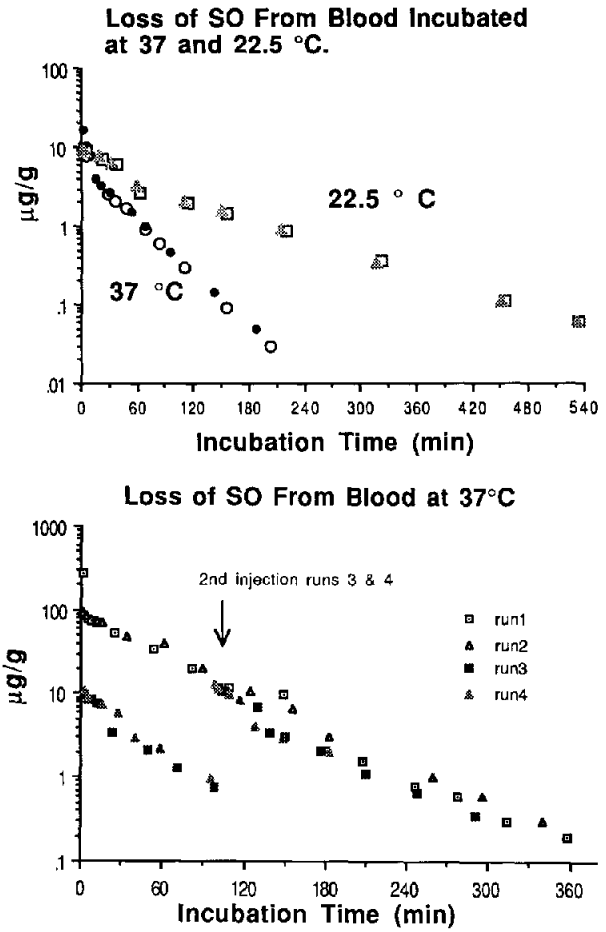

Fig. 3. Disappearance of styrene-7,8-oxide from whole rat blood. In experiment I SO was added at 10 µg/g to blood at room temperature (22.5°C) and at rat body temperature (37°C). In experiment II SO was added at 10 and 100 µg/g to blood at rat body temperature. Note in this experiment a second 10 µg/g SO spike was made in the lower level vials when the SO concentration had dropped to about 1 µg/g.

addition was made. The second SO addition was used to evaluate the potential for time-dependent changes in factors affecting SO disappearance. The calculated half-life for the 100 µg/g level was 41 min while that for the first phase of the 10 µg/g level was 27 min. The half-life of the 10 µg/g level from the second SO addition on was 34 min, not considered significantly different than the first addition.

Using the method described here, SO could readily be observed in the blood of rats given oral doses of SO equivalent to those used previously in a chronic toxicity study [9]. Fig. 4 depicts the mean concentration of SO in the blood of rats given single oral doses of 275 or 550 mg/kg SO. The primary observation is that blood SO levels were much higher than expected (*i.e.*, in the ppm range) given the

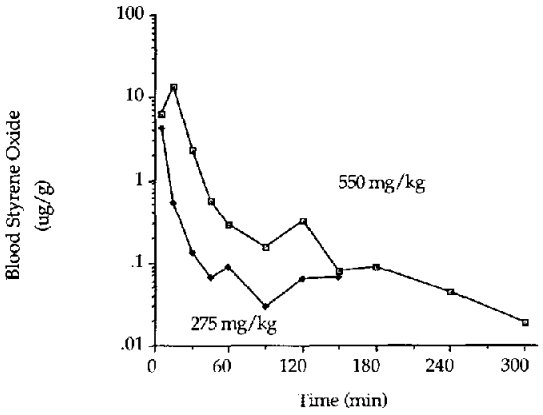

Fig. 4. Blood styrene oxide levels in Fischer rats given a single oral dose of 275 or 550 mg/kg styrene oxide.

labile nature of this chemical. It should be noted, however, that individual animal blood SO levels (data not shown) did not exhibit a consistent pattern across time and were extremely variable between animals. For example, the highest concentrations of SO in the rats given the 275 and 550 mg/kg dose level ranged from 0.27 to 8.84 μg/ml and 2.1 to 32.4 μg/ml, respectively. The variability in blood SO levels is consistent with the slow and variable absorption of a material that is rapidly metabolized. Fasting the rats overnight prior to dosing may have reduced the variability in absorption but was not done because the animals were not fasted in previously reported toxicity studies with SO [9].

The areas under the blood SO concentration time curve (AUC), calculated using the trapezoidal were 47 and 286 min μg/g for the 275 and 550 mg/kg dose levels, respectively. These areas were not proportional to the dose and suggest that the elimination of SO may have been partially saturated at the 550 mg/kg dose level.

In conclusion, a method has been developed for the direct determination of SO in whole rat blood with a detection limit of 0.01 μg/g. This method requires only 0.1 ml of blood and is ideally suited for use in kinetic studies involving rodents. In addition, because the sample preparation is simple, the analysis is automated, and the run-times are short, this method lends itself to studies generating large numbers of samples. The kinetics experiments presented confirm that rate of disappearance of SO from whole rat blood is slow enough to allow accurate, reproducible measurement of SO in blood using this method.

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