Scand J Work Environ Health 1984;10(3):179-187

<https://doi.org/10.5271/sjweh.2345>

Issue date: Jun 1984

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This article in PubMed: www.ncbi.nlm.nih.gov/pubmed/6474112

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Occurrence of styrene-7,8-oxide and styrene glycol in mouse after the administration of styrene

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LÖF A, GULLSTRAND E, LUNDGREN E, BYFÄLT NORDQVIST M. Occurrence of styrene-7,8-oxide and styrene glycol in mouse after the administration of styrene. *Scand J Work Environ Health* 10 (1984) 179–187. Styrene-7,8-oxide and its hydrated product styrene glycol were determined in mouse tissues at different times (0.5–5 h) after the intraperitoneal administration of 7-[¹⁴C]-styrene (3.8 mmol/kg). In a study of the influence of dose on the metabolite pattern of styrene, mice were killed 2 h after a dose of 1.1, 2.3, 3.4, and 5.1 mmol/kg, respectively. The mouse tissues studied (blood, liver, kidney, lung, brain, subcutaneous adipose tissue) were isolated and extracted first with hexane to remove styrene and styrene-7,8-oxide and then with ethyl acetate to remove styrene glycol. β -Glucuronidase was used to liberate conjugated styrene glycol. A gas-liquid chromatographic method based on the use of an electron capture detector (GLC-EC) was used to quantify styrene glycol, as well as styrene-7,8-oxide, after hydrolysis. In addition all homogenates and extracts were assayed by radioactivity counting. Styrene-7,8-oxide and styrene glycol reached maximum concentrations within 2 h. The highest levels of styrene-7,8-oxide were detected in the kidneys and subcutaneous adipose tissue, while the lungs showed the lowest levels. Styrene glycol was found in the highest concentrations in the kidneys, liver, blood, and lungs. The concentration of unmetabolized styrene increased exponentially at higher doses. There seemed to be a linear increase with the dose of styrene-7,8-oxide and styrene glycol in all the tissues studied. The more polar metabolites occurred at relatively lower levels in the liver and kidneys at higher doses. In a complementary study the epoxide hydratase inhibitor trichloropropene oxide was added to the removed tissues, and the hexane extracts were analyzed for styrene-7,8-oxide both by GLC-EC and mass spectrometry (GLC-MS).

Key terms: dose dependence, electron capture detector, gas chromatography, mass spectrometry, metabolism, radiolabeled chemical, tissue distribution.

Styrene (vinylbenzene, phenylethylene) is one of the major compounds in the production of plastic materials. The worldwide production of the monomer is about $7 \cdot 10^6$ tons per year (29). Especially workers manufacturing glass-reinforced plastics are exposed to styrene as it evaporates during the laminating process. Human exposure to styrene mainly occurs via inhalation (34), and absorbed styrene is, according to animal studies, rapidly distributed in the body (4, 37). A predominant part is distributed in a metabolized form (16).

The metabolism of styrene has been extensively studied, and epoxidation at the -7,8- position has been suggested as the major metabolic pathway (14, 15, 20, 22, 33, 35), a reaction preferentially catalyzed by microsomal cytochrome P-450 (14, 30). Styrene-7,8-oxide (phenyloxirane) is hydrated to styrene glycol (1-phenyl-1,2-ethanediol) by microsomal epoxide hydratase (19, 33). Styrene glycol can be conjugated with β -glucuronic acid (12, 21) or oxidized to mandelic, phenylglyoxylic, and benzoic acid [see Löf et al

(16)]. Another prominent pathway of styrene metabolism is the conjugation of styrene-7,8-oxide with glutathione catalyzed by glutathione S-transferases (6, 25, 26). The glutathione conjugates are then degraded to mercapturic acids before excretion (13, 27, 28). Ring hydroxylation of styrene occurs to a small extent, as does the formation of phenyl ethanol [see Löf et al (16)].

Metabolism is important in the understanding of the toxicity of styrene. Epoxide hydratase and glutathione S-transferase may play protective roles with regard to the toxic effects of styrene-7,8-oxide (22, 30). The lung is the only organ that shows higher activity for the monooxygenase system than for epoxide hydratase in vitro (7), a phenomenon indicating the risk of a pulmonary accumulation of styrene-7,8-oxide. The biologically active styrene-7,8-oxide has been found in vitro as a styrene metabolite in rat liver microsomes (3, 14, 33) and in isolated perfused rat liver (1). Styrene-7,8-oxide can also be formed by human erythrocytes and lymphocytes (2, 18). It has furthermore been detected in vivo as a styrene metabolite in trace amounts in the lungs and liver of mice pretreated with an inhibitor of epoxide hydratase (22). Recently we reported the occurrence of styrene-7,8-oxide in human blood (35), and in the blood and other tissues of mice (17). After the initial findings of

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conjugated styrene glycol in rat urine (12, 21) styrene glycol has been determined as an *in vivo* metabolite of styrene in mouse tissues (16) and in blood from experimentally exposed volunteers and occupationally exposed workers (35, 36). The main purpose of the present study was to detect styrene-7,8-oxide and, if possible, quantify this epoxide and its importance in the metabolism of styrene.

Materials and methods

Radioactively labeled 7-[^{14}C]-styrene (The Radiochemical Centre, Amersham, England; radiochemical purity 98 % and chemical purity 99 %) with a specific activity of 3.95 MBq/mmol was mixed with dimethyl sulfoxide (Merck, analytical grade) and corn oil and used as the dosing solution. In the determination of the occurrence of styrene and its metabolites in time in different tissues, groups of four male NMRI (Naval Medical Research Institute) mice (25–30 g) were injected intraperitoneally with styrene (3.8 mmol/kg). The groups were killed after 0.5, 1, 2, and 5 h, respectively. In the study of the influence of the dose of styrene, groups of four mice were killed 2 h after a dose of 1.1, 2.3, 3.4, and 5.1 mmol/kg, respectively. Blood, liver, kidneys, lungs, brain, pancreas, and subcutaneous adipose tissue were isolated, weighed, and homogenized in 0.1 M phosphate buffer (pH 7.4), except for the adipose tissue which was homogenized in hexane with the phosphate buffer added afterwards. The homoge-

nized tissues were first extracted twice with hexane (Merck, analytical grade) to remove styrene and styrene-7,8-oxide (figure 1, step 1) and then twice with ethyl acetate (Merck, analytical grade) to remove styrene glycol (figure 1, step 2). Conjugated metabolites were cleaved with β -glucuronidase type H-1 (Sigma) in 0.07 M acetate buffer pH 5.0 at 37°C overnight and extracted twice with equal volumes of ethyl acetate. Acidic metabolites were extracted twice with equal volumes of ethyl acetate after acidification to pH 3. The radioactive contents of the homogenates and the hexane and ethyl acetate extracts, as well as of the residual aqueous phases, were quantified by liquid scintillation counting (16). The radioactivity was assayed in a Packard Tri Carb model 3375 spectrometer with external standardization. The radioactivity of the hexane extracts was considered to be equivalent to the contents of unchanged styrene. The standard error of the method as determined on liver samples was 8 % in the homogenate, 12 % for styrene in the hexane extracts, 9 % in the ethyl acetate extracts, and 6 % for the polar metabolites in the aqueous phase remaining after all extractions.

The contents of the nonconjugated and enzymatically liberated styrene glycol in the ethyl acetate extracts were determined by gas-liquid chromatography with an electron capture detector (GLC-EC) (3 % SE-30 on Chromosorb GAW-DMCS, 2.0 m, 240°C, nitrogen flow 40 ml/min, Carlo Erba FTV 2350) after derivatization with pentafluorobenzoyl chloride (Aldrich 98 %). Allylbenzene glycol, synthesized according to Duverger-van Bogaert et al (11), was added as the internal standard prior to the extractions with ethyl acetate. The peak areas of derivatized styrene glycol and allylbenzene glycol were integrated (Varian Vista 401 Chromatography Data System). Calibration curves were obtained after the addition of 10- μl toluene solutions of styrene glycol (Aldrich 97 %) to blood and tissues after homogenization and extraction with hexane. The concentration range was 0–100 $\mu\text{mol/l}$ for styrene glycol. The calibration curves were fitted to a double logarithmic relation and could be applied down to a concentration of approximately 1 $\mu\text{mol/l}$. The standard error of the method as determined on liver samples was 10 %.

Styrene-7,8-oxide was quantified in the hexane extracts after hydrolysis to styrene glycol with sulfuric acid (0.5 M) and the addition of allylbenzene glycol as the internal standard (figure 1, step 3). Calibration curves were obtained after the addition of 10- μl toluene solutions of styrene-7,8-oxide (Fluka AG, Buchs SG 97 % purity, distilled) to blood and tissues after homogenization. The concentration range was 0–20 $\mu\text{mol/l}$ for styrene-7,8-oxide. The calibration curves were linear for the concentration range in question and could be applied down to a concentration of approximately 0.2 $\mu\text{mol/l}$. The calibration curves for styrene-7,8-oxide in the blood, pancreas, lungs, and brain were considered equivalent [slope (k) = 0.69].

Figure 1. A scheme for the extraction of styrene and styrene-7,8-oxide (step 1) and styrene glycol (step 2) from tissue homogenates. Allylbenzene glycol (ABG) was added as the internal standard. Styrene-7,8-oxide was hydrolyzed with sulfuric acid (H_2SO_4) to styrene glycol (step 3). The ethyl acetate extracts were derivatized with pentafluorobenzoyl chloride and analyzed by gas-liquid chromatography with an electron capture detector. (NaOH = sodium hydroxide)

The slope of the corresponding curve for the kidney was lower ($k = 0.58$) and that of the curve for subcutaneous adipose tissue higher ($k = 0.97$). For the liver the slope of the calibration curve was extremely low ($k = 0.11$). The possibility of styrene glycol contamination in the hexane extracts was examined, and a maximum of 0.3 % of styrene glycol added to homogenates was recovered in the hexane extracts. The standard error of the method as determined on liver samples was 15 %.

In a complementary study the epoxide hydratase inhibitor trichloropropene oxide (Aldrich 98 %) was added to a concentration of 10 mM prior to the homogenization to mouse tissues removed 30 min after the intraperitoneal administration of styrene (2.7 mmol/kg). Aliquots of the hexane extracts were analyzed with the use of GLC-EC as already described or with a gas chromatograph with a mass spectrometer (Hewlett Packard 5985 A) (GC-MS) equipped with a capillary column (silica, 0.2 mm $\times$ 12 m). The column temperature was 70°C, and the electron energy 70 eV. Selected ions were monitored at m/e 120 (M^+) and 91 (5, 33).

The standard errors of the methods were determined according to

$$SE = \{[\Sigma d^2 - (\Sigma d)^2/n]/2(n-1)\}^{1/2},$$

where d is the difference in concentration between duplicate samples.

Results

The accumulation of total radioactivity after the intraperitoneal administration of 7- ^{14} C-styrene agreed well with the results of our earlier study (16). The concentrations of styrene are given in figure 2. Blood, lungs, and brain showed the lowest concentrations of styrene, while liver, kidneys, and pancreas had higher concentrations. Subcutaneous adipose tissue (not in figure 2) had the highest concentration, 8.5 μ mol of styrene/g of tissue after 2 h and 0.9 μ mol of styrene/g after 5 h. In the previous study the styrene concentrations showed the highest values after 30 min, whereas in the present study the concentrations after 30 and 60 min were about the same. The concentration of unmetabolized styrene at a time delay of 2 h seemed to increase exponentially with the dose in all tissues except pancreas (figure 3). The styrene concentration in subcutaneous adipose tissue (not in figure 3) also seemed to increase exponentially to 12.5 μ mol/g after the highest dose given.

The sum of free and conjugated styrene glycol (as determined by GLC-EC) at different time delays is given in figure 4. A maximum appeared in all tissues 2 h after the administration. The kidneys showed the highest maximum, and subcutaneous adipose tissue the lowest. In the kidneys a prominent part of the styrene glycol occurred bound to β -glucuronic acid or sulfate (table 1). Also in the blood, liver and lungs,

Figure 2. Tissue concentrations of unmetabolized styrene as a function of time after the intraperitoneal administration of 7- ^{14} C-styrene (3.8 mmol/kg). The concentration of styrene is expressed as micro-moles of styrene per gram of wet tissue. Mean values ($N = 4$) and standard deviations are shown.

the conjugated fraction exceeded that in other tissues. There was a tendency towards a higher fraction of bound styrene glycol after a longer time delay and after a lower dose. No tendency towards a decreased relative occurrence of styrene glycol at higher doses was observed (figure 5). The concentrations of styrene glycol determined by the GLC-EC method correlated well with the data received from liquid scintillation counting (correlation coefficient = 0.93).

The highest concentration of styrene-7,8-oxide was found in subcutaneous adipose tissue with a maximum after 2 h (figure 6). In the kidneys the concentration of styrene-7,8-oxide was comparatively high

already after 30 min. In blood and liver the maximal concentration of styrene-7,8-oxide seemed to appear after 2 h. The concentration of styrene-7,8-oxide in the lungs was very low. There was probably a linear increase in styrene-7,8-oxide with the dose in all the tissues studied (figures 7 & 8).

When an epoxide hydratase inhibitor was added, the relative concentrations of styrene-7,8-oxide in different tissues, as determined by the two methods of analysis (GLC-EC, GLC-MS), were in good agreement (table 2). The extractable contents of styrene-7,8-oxide in the liver and lungs increased considerably when the homogenization was performed in the

Figure 3. Tissue concentrations of un-metabolized styrene as a function of dose 2 h after the intraperitoneal administration of 7-[¹⁴C]-styrene. The concentration of styrene is expressed as nanomoles of styrene per gram of wet tissue. Mean values (N = 4) and standard deviations are shown.

Table 1. Percentage of styrene glycol conjugated with β -glucuronic acid or sulfate. The styrene glycol was analyzed by gas chromatography with an electron capture detector and by liquid scintillation counting. Mean values (N = 4) and standard deviations are shown.

	Styrene glycol (%)							
	Blood		Liver		Kidneys		Lungs	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Time after intraperitoneal administration of a 7-[¹⁴ C]-styrene dose of 3.8 mmol/kg								
0.5 h	13.7	6.0	20.0	7.5	36.5	15.7	14.0	8.3
1—2 h	22.9	8.3	25.6	5.8	43.5	7.7	19.4	8.7
5 h	34.4	5.2	34.4	9.6	59.3	6.8	26.3	5.6
Dose of 7-[¹⁴ C]-styrene administered intraperitoneally (styrene glycol percentage determined 2 h after administration)								
1.1 mmol/kg	38.0	8.1	31.9	8.4	56.5	8.1	32.3	5.8
2.3—3.4 mmol/kg	23.8	6.1	24.5	2.4	42.3	8.2	19.6	7.4
5.1 mmol/kg	27.4	3.8	26.6	1.4	40.0	3.9	15.7	3.8

Figure 4. Sum of free and enzymatically liberated styrene glycol in different tissues as a function of time after the intraperitoneal administration of 7-[^{14}C]-styrene (3.8 mmol/kg). The concentration of styrene glycol is expressed as nanomoles of styrene glycol per gram of wet tissue. Mean values ($N = 4$) and standard deviations are shown. (subc adip = subcutaneous adipose)

Figure 5. Sum of free and enzymatically liberated styrene glycol in different tissues as a function of dose 2 h after the intraperitoneal administration of 7-[^{14}C]-styrene. The concentration of styrene glycol is expressed as nanomoles of styrene glycol per gram of wet tissue. Mean values ($N = 4$) and standard deviations are shown. (subc adip = subcutaneous adipose)

Figure 6. Tissue concentrations of styrene-7,8-oxide as a function of time after the intraperitoneal administration of 7-[^{14}C]-styrene (3.8 mmol/kg). The concentration of styrene-7,8-oxide is expressed as nanomoles of styrene-7,8-oxide per gram of wet tissue (Mean values ($N = 4$) and standard deviations are shown. (subc adip = subcutaneous adipose)

Table 2. Tissue:blood concentration ratio of styrene-7,8-oxide 30 min after the intraperitoneal administration of styrene (2.7 mmol/kg) as determined by two different techniques. (GLC-EC = gas-liquid chromatography-electron capture detector, GLC-MS = gas-liquid chromatography-mass spectrometry, subc adip = subcutaneous adipose)

	subc adip tissue	>liver	>lungs	>kidneys	>blood
GLC-EC	19	8	4	2	1
GLC-MS	14	7	3	2	1

presence of the epoxide hydratase inhibitor. However, a more thorough investigation must be done to evaluate the role of the inhibitor.

The radioactivity remaining in the aqueous phase after the extraction of the acids with ethyl acetate at pH 3 is depicted in figure 9. The kidneys and liver exhibited the highest concentrations of these hydrophilic metabolites, the blood and brain the lowest. There was a linear increase of residual aqueous radio-

Figure 7. Tissue concentrations of styrene-7,8-oxide as a function of dose 2 h after the intraperitoneal administration of 7-[¹⁴C]-styrene. The concentration of styrene-7,8-oxide is expressed as nanomoles of styrene-7,8-oxide per gram of wet tissue. Mean values (N = 4) and standard deviations are shown.

Figure 8. The concentration of styrene-7,8-oxide in subcutaneous (subc) adipose (adip) tissue as a function of dose 2 h after the intraperitoneal administration of 7-[¹⁴C]-styrene. The concentration of styrene-7,8-oxide is expressed as nanomoles of styrene-7,8-oxide per gram of wet tissue. Mean values (N = 4) and standard deviations are shown.

Figure 9. Tissue concentrations of hydrophilic metabolites as a function of dose 2 h after the intraperitoneal administration of 7-[¹⁴C]-styrene. The concentration is expressed as micromoles per gram of wet tissue. Mean values (N = 4) and standard deviations are shown.

activity in the blood, lungs, pancreas, and brain. In the kidneys and the liver the occurrence of such an increase leveled off at higher doses.

Discussion

Styrene and its metabolites were rapidly distributed in the tissues as has earlier been discussed in detail (16). A possible explanation of the linear dose-response curve for styrene in the pancreas (figure 3) may be related to the route of administration, whereas the deviation from linearity seen for the other tissues may reflect a diminished metabolism at higher doses. Another explanation could be decreased rates of distribution, but this possibility is opposed by the linearity of the styrene concentration in pancreas. Dose-dependent kinetics have been observed earlier for biphasic styrene elimination in the rat (37, 38). After exposure to between 200 and 600 ppm for 6 h (blood concentrations comparable with ours) the clearance of styrene in the rat became saturated and resulted in a higher steady-state level in blood at higher doses (24). This phenomenon can mainly be ascribed to a limited metabolic capacity of the cytochrome P-450 system.

The contents of styrene-7,8-oxide were calculated from individual calibration curves for the different tissues. The low slope of the calibration curve for liver, about 15 % of that of the blood curve, made the quantification of styrene-7,8-oxide in the liver rather uncertain. The high hepatic activity of epoxide hydratase probably resulted in an extensive conversion of styrene-7,8-oxide to styrene glycol during the isolation work. This occurrence was confirmed after the addition of the epoxide hydratase inhibitor trichloropropene oxide to the excised liver prior to the homogenization. The standard curve so obtained was similar to those of the other tissues, which equaled a standard curve made in phosphate buffer. The simultaneous analysis of hexane extracts by GLC-EC and by GLC-MS verified the identification of styrene-7,8-oxide.

The highest concentration of styrene-7,8-oxide was found in subcutaneous adipose tissue (figures 6 & 8), a result indicating an accumulation of the epoxide in the adipose tissue. Bidoli et al (5) reported a half-time of 3.4 min for styrene-7,8-oxide in mouse blood *in vivo*. Accordingly it is possible for the styrene-7,8-oxide to be transported to the adipose tissue, as well as to other tissues. Styrene-7,8-oxide may also be formed at the site, eg, by lipid peroxides (3). On the basis of the monooxygenase activities the highest formation of styrene-7,8-oxide would be expected in the liver followed by the lungs and kidneys (7). The high concentration of styrene-7,8-oxide in the kidneys already after 30 min (figure 6) points to a formation at that site. The relatively high hepatic content of styrene-7,8-oxide after 5 h and the low slope of the calibration curve for liver indicate that the concentra-

tion of styrene-7,8-oxide in the liver is probably overestimated. Despite a reported higher *in vitro* activity for the monooxygenase system than for epoxide hydratase, we found very little styrene-7,8-oxide in the lungs (7). In the complementary study with the epoxide hydratase inhibitor the estimated concentration of styrene-7,8-oxide increased considerably in the liver and the lungs. The explanation for this phenomenon is not known.

The dose-response curves for styrene-7,8-oxide are probably linear, with possible exceptions of those for the kidneys and subcutaneous adipose tissue, in which the elimination of the epoxide might be less effective at higher doses (figures 7 & 8). This finding may indicate a potential risk for renal tissue damage after styrene administration at high doses. It was recently shown in mice that the occurrence of single-strand breaks increased in deoxyribonucleic acid (DNA) from the kidneys after the intraperitoneal administration of increased doses of styrene and styrene-7,8-oxide, respectively (32). The levels of single-strand breaks were higher in the kidneys than in the other tissues after styrene administration.

No dose-dependent decrease in the conversion of styrene to styrene glycol could be verified (figure 5). As in our previous study (16) the highest concentration of styrene glycol was found in the kidneys with a prominent fraction conjugated, a phenomenon reflecting renal excretion. The occurrence of styrene glycol in the brain is interesting as this substance has been proclaimed to be a central nervous system depressant (23), but some remains of blood in the brain samples cannot be excluded. Comparatively high levels of single-strand breaks in brain tissue DNA after styrene administration have been reported (32).

The concentration of styrene-7,8-oxide in the blood was approximately 2 % of that of styrene glycol 1–2 h after the administration of a styrene dose of 3.8 mmol/kg. This result is in agreement with the relative concentrations in human blood at the end of a 2-h inhalation exposure when the level of styrene glycol is approximately 15 % of the styrene concentration (35). The amount of conjugated styrene glycol as the percentage of the total amount of styrene glycol in human blood was 26.1 (SD 6.4) % after inhalation exposure (34); this value is in agreement with the conjugated fraction in mouse blood (table 1).

The radioactivity remaining in the aqueous phase can be considered to consist of glutathione conjugates. This hypothesis will be discussed further in a separate paper (to be published). The glutathione conjugates did not appear to the same extent in the liver and the kidneys, the organs with the highest contents of hydrophilic metabolites, after the highest doses of styrene (figure 9). A dose dependence in both glutathione conjugation and the biliary excretion of styrene metabolites has earlier been indicated

in studies with rats (8, 9, 10, 31). A diminished metabolism of styrene-7,8-oxide to glutathione conjugates may increase the amount of the epoxide and thus the toxicity of styrene. In the kidneys the occurrence of this phenomenon was supported by the seemingly exponential rise of styrene-7,8-oxide with the dose. In the liver it could not be verified.

Acknowledgments

The authors are very grateful to Ms J Gorczak for her skillful help with the mass spectrometric analysis and Ms MB Cedervall for her patient typing of this manuscript.

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Received for publication: 27 February 1984