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Note

Determination of styrene in biological samples by reversed-phase liquid chromatography

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Styrene, a monomer widely used in the plastic and synthetic rubber industry [1], is known to have hepatorenal toxic properties [2] The determination of this monomer in blood and target organ tissues is needed for toxicokinetic and distribution studies. A few chromatographic methods have been published for the determination of the styrene monomer in air, food and biological samples [3—7] Most of them have used gas chromatography (GC) with electron-capture, flame ionization or mass spectrometric (MS) detection Determination of styrene in whole blood and tissue homogenates has been studied by Karbowski and Braun [3] using GC—MS (selected-ion monitoring) to assay the styrene monomer from hexane extracts with a sensitivity ranging from 2 to 200 ng/g depending on the tissue The high blood volume (5 ml) necessary for the extraction procedure makes this method unsuitable for kinetic studies in small animals. Other investigators, like Withey and Collins [4] and Hoshika [5] proposed other GC methods, one using an unusual headspace technique, the other requiring derivatization with bromine prior to analysis A very few high-performance liquid chromatographic (HPLC) procedures have been proposed until now. Lof et al. [6] were able to separate and quantify radiolabeled

0378-4347/86/$03 50

styrene and styrene glycol with a Micropak MCH-10 reversed-phase column eluted with a methanol—water linear gradient The need for a gradient device and multiple ethyl acetate extractions limit the use of this method Finally, Gawell and Larsson [7] described an HPLC assay for styrene in foods, involving azeotropic distillation with methanol before HPLC analysis Variable recoveries were obtained according to the concentration in 50-g samples, and the internal standard was added after the azeotropic distillation. Therefore, the lack of a simple and sensitive method to assay styrene in biological samples prompted us to develop a rapid reversed-phase HPLC procedure using benzene as an internal standard.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile, benzene and toluene were supplied by BDH Chemicals (Toronto, Canada). Styrene, with a purity of 99%, was obtained from Fisher Scientific (Montreal, Canada) and was stabilized with 0 003% *tert* -butylcatechol Anhydrous dibasic potassium phosphate, purchased from Fisher Scientific, was reagent grade. Spectro-grade *p*-diethylbenzene with a purity of 98% was supplied by Aldrich (Milwaukee, WI, U S A).

Apparatus

A Varian Model 5010 HPLC apparatus equipped with a Varian Varichrom variable-wavelength detector was connected to a Varian Model G 2500 recorder (Varian, Palo Alto, CA, U S A) A 15 cm × 3 9 mm I D stainless-steel Nova-Pak column prepacked with an octadecyl silica phase (5 μm mean particle size) was supplied by Waters Assoc (Milford, MA, U S A.) A Rheodyne (Cotati, CA, U S.A.) 1-μm filter was installed between the Valco loop injector (Varian) of the chromatograph and the analytical column

Preparation of samples and extraction

Serum was obtained from centrifuged blood (500 *g* for 10 min) collected from anesthetized 250-g female Sprague—Dawley rats (Charles River, St Constant, Canada) which were sacrificed for removal of liver and kidneys. The organs were homogenized in potassium chloride (1.15%) with a Brinkmann Polytron® apparatus (Brinkmann Instruments, Westbury, NY, U S.A.) The homogenates contained 250 mg of organ tissue per ml.

To glass extraction tubes, 100 μl of either serum, liver or kidney homogenate were added Enough dibasic potassium phosphate (approx 50 mg) to saturate the volume of biological sample was added to increase the styrene extractability and effect the separation from the extraction solvent Then, 1 ml of acetonitrile containing 439.35 μg of benzene as chromatographic standard (this is corresponding to its 1 2000 dilution in acetonitrile) was added, and extraction and protein precipitation were processed by vortexing for 30 s Centrifugation at 500 *g* for 5 min followed, and 50 μl of the organic phase (upper layer) were injected directly into the chromatograph

Chromatographic conditions and UV detection

The mobile phase, filtered on a 0 45-μm Millipore® filter (Toronto, Canada)

prior to use, was acetonitrile—water (60 40) The elution was maintained at a constant flow-rate of 1.3 ml/min Detection wavelength was fixed in the UV range at 245 nm, where the absorbance index of styrene is at a maximum, with a sensitivity ranging from 0 2 to 0.05 a.u.f s The recorder was set to give a full scale deflection at 1 mV. Chart speed was fixed at 0.1 in /min to allow optimal peak shape.

Recovery

Aliquots of serum and tissue homogenate were spiked with known quantities of styrene (five determinations at 0.1, 3.6 and 180 μg/ml in each case). Thereafter, the samples were extracted and chromatographed as described above and the peak heights for styrene were compared with the peak heights obtained from a standard acetonitrile solution of styrene

Quantitation

Standard curves for styrene were constructed using peak-height ratios obtained for the monomer to the internal standard versus the concentration of the monomer. The concentration of styrene in biological samples was determined by interpolation following linear regression of the standard curve All results are expressed in terms of arithmetic mean, $\bar{x}$, and standard error, S.E.

RESULTS AND DISCUSSION

Retention times and background

Fig 1 shows a typical chromatogram from a liver homogenate extract. Retention times for styrene and the chromatographic internal standard benzene are approximately 3 2 and 2.2 min, respectively. Total time for analysis is less than 4 4 min

Several xenobiotic-free samples of serum and liver or kidney homogenates were processed as described above. Background peak interference at the elution times corresponding to the compounds tested was found to be absent, as shown in Fig 1 for liver homogenate samples

Standard curves

Standard curves were made from serum and liver or kidney homogenates, spiked with known quantities of styrene The use of concentrations in the range 0.05- 180 μg/ml of serum showed a good linear correlation ($r^2 > 0.99$) with minimal y-intercept (Table I). These concentrations (0 05, 1 2, 3 6, 7 2, 18 0, 36 0, 60 0 and 180 0 μg/ml of serum, liver or kidney homogenate) were chosen based on animal experiments in which Sprague—Dawley rats received a toxic dose of 800 mg/kg styrene by the intraperitoneal route, with collection of blood and removal of liver and kidneys 1, 2 and 4 h post-administration This protocol was representative of toxicokinetic studies under development in our laboratory The concentration range for tissue homogenates was 0 2—720 μg of styrene per g of liver or kidney Also, very good linearity was found in this case (Table I).

Recovery and sensitivity

The analysis of spiked samples of serum and liver or kidney homogenate

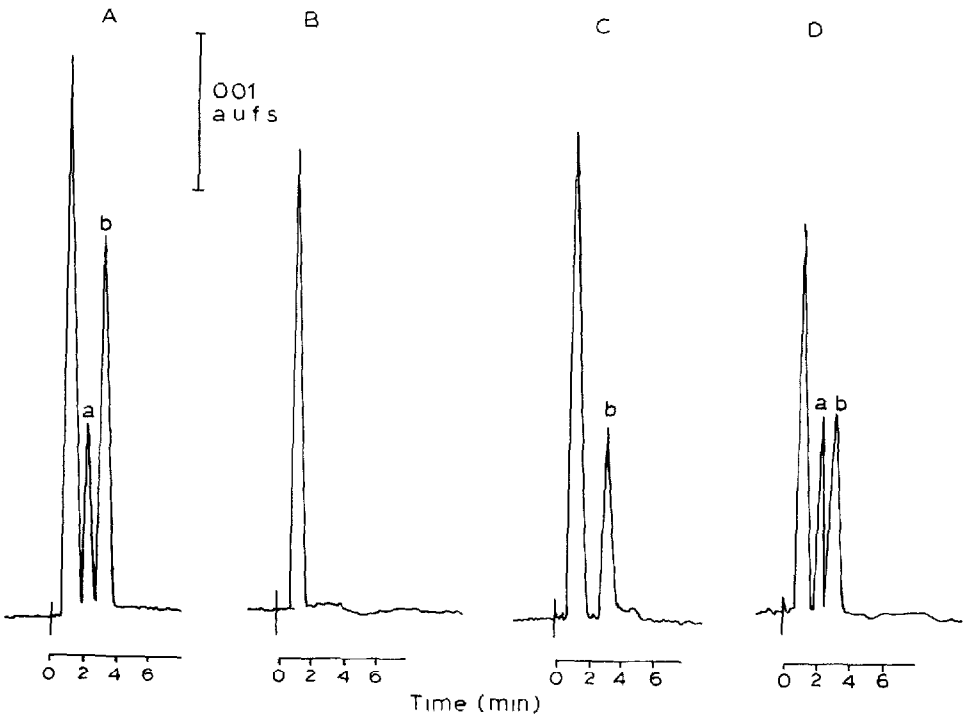

Fig 1. Chromatograms of (A) extracted spiked liver homogenate sample containing styrene at a concentration of 22 5 μg/ml and benzene at an equivalent concentration of 4393 5 μg/ml, (B) extracted styrene- and benzene-free liver homogenate, (C) an extract of liver homogenate sample obtained from a rat after the intraperitoneal administration of a single dose of styrene (500 mg/kg), (D) an extract of the preceding liver sample (chromatogram C) containing the chromatographic standard Peaks a = benzene, b = styrene, respectively

TABLE I

LINEAR REGRESSION ANALYSIS OF STANDARD CURVES (n = 3)

Sample	Equation of straight lines for eight standard samples	r^2
Serum	$y = (0\ 0382 \pm 0\ 0011)x^{*} + (0\ 1861 \pm 0\ 0058)$	0 998
Liver	$y = (0\ 0058 \pm 0\ 0003)x^{**} + (0\ 0773 \pm 0\ 0039)^{***}$	0 998
Kidney	$y = (0\ 0060 \pm 0\ 0003)x^{**} + (0\ 0472 \pm 0\ 0023)^{***}$	0 998

*x in μg/ml
**x in μg/g considering 250 mg of tissue per ml of homogenate
***The difference in the y-intercept for the liver and kidney homogenates is statistically insignificant ($p < 0\ 01$)

gave a recovery independent from the added amount of styrene The recovery from these samples was complete, with a mean value of 106.5 ± 2 5% The respective recoveries at 180, 3 6 and 0 1 μg/ml of biological sample were 101.3 ± 2 5, 106.5 ± 2 6 and 102 1 ± 2 5% in the serum, 104.2 ± 2 6, 109 5 ± 2.7 and 108 7 ± 3.0% in the liver homogenate and 107 3 ± 2 6, 112 5 ± 2.8 and 106.5 ± 2.1% in the kidney homogenate Analysis of variance of these data indicated that the recovery was independent of the concentration and of the biological sample ($p > 0.05$).

Sensitivity of our method, with the instrumentation used, was estimated to be 50 ng/ml of serum or tissue homogenate based on a signal-to-noise ratio of 5 In this latter case, because the concentration is 250 mg of organ tissue per ml of homogenate, it means that sensitivity is 200 ng/g of tissue Lower values could probably be reached by using homogenates of higher tissue concentration The limit of detection was 1 ng/ml of acetonitrile solution with a signal-to-noise ratio of 2

Precision and accuracy

To determine the within-day and between-day assay precision for serum and tissue homogenates, replicates at different styrene levels were analyzed The reproducibility is summarized in Table II and is found to be very satisfactory, as indicated by the low values of the coefficients of variation (C V.)

Accuracy of the method was evaluated by spiking biological samples with five known amounts of styrene (but different from the ones used for the standard curves) and by calculating the deviation between theoretical and determined concentrations As shown in Table III, low values for the percentage of deviation were found for serum and liver or kidney homogenates

TABLE II

WITHIN-DAY AND BETWEEN-DAY PRECISION FOR THE DETERMINATION OF STYRENE CONCENTRATION IN RAT SERUM AND LIVER OR KIDNEY HOMOGENATES

Theoretical concentration	Coefficient of variation* (%)	
	Within-day (n = 5)	Between-day (n = 3)
Serum (μg/ml)		
180 0	3 1	-
18 0	1 7	3 1
3 6	2 9	2 6
0 5	4 0	5 0
0 1	9 2	5 5
Liver homogenate (μg/g)		
720 0	3 8	-
144 0	4 1	6 8
14 4	2 6	3 4
2 0	4 5	4 5
0 4	4 2	5 0
Kidney homogenate (μg/g)		
720 0	5 2	-
144 0	4 5	6 8
14 4	2 3	3 7
2 0	4 2	6 0
0 4	4 2	6 0

*Coefficient of variation of peak-height ratios

TABLE III

ACCURACY OF THE DETERMINATION OF STYRENE CONCENTRATION IN RAT SERUM AND LIVER OR KIDNEY HOMOGENATES

Theoretical concentration (μg/ml)			Deviation (%)		
Serum	Liver	Kidney	Serum	Liver	Kidney
90 0	360 0	360 0	7 8	7 6	4 7
45 0	180 0	180 0	4 3	3 5	4 7
22 5	90 0	90 0	5 7	10 0	6 3
11 2	45 0	45 0	5 9	6 4	5 3
5 6	22 5	22 5	5 9	6 3	2 5
0 9	0 9	0 9	4 0	5 2	5 6
0 45	0 45	0 45	6 1	6 0	5 0
0 225	0 225	0 225	7 2	6 7	7 0
0 1	0 1	0 1	8 0	8 5	7 9
			6 1 ± 0 4*	6 7 ± 0 6*	5 4 ± 0 5*

*Arithmetic mean of deviation ± standard error

Interference and application

None of the following solvents were found to interfere with this assay acetone, ethylacetate, *n*-hexane and monochlorobenzene

The use of benzene as a chromatographic standard was chosen because of structural similarity with styrene and shorter retention time Diethylbenzene could also be used as a chromatographic standard, with a retention time of 4 3 min, if benzene had to be assayed also or represented a significant interference with styrene in some experimental exposure experiments. The recovery of this chromatographic standard from a spiked rat serum sample (100 μg/ml) was found to be excellent too 101 3 ± 1 53% for $n = 3$. Also, chromatographic separation of styrene, benzene and toluene (another toxic solvent) can be obtained by using a mobile phase of water—acetonitrile (60 40) at a flow-rate of 1 0 ml/min and diethylbenzene as chromatographic standard In these experimental conditions, the retention times of styrene, benzene, toluene and diethylbenzene were found to be 6 4, 3 5, 5 2 and 8 0 min, respectively, and the total analysis time would be 9 min This would be therefore very useful in separating each solvent when these are present in a mixture.

On the other hand, the volume of serum used in our method can be decreased further to at least 50 μl without modifying the recovery in the extraction or the reproducibility or accuracy as we have found before. This low volume of sample is obviously important when small animals like mice are used in kinetic studies With this low sampling volume, the recovery in the extraction of styrene from samples of serum, liver or kidney homogenate in the concentration range 0 1—180 0 μg/ml was 104 6 ± 1 2% ($n = 9$), 102 9 ± 1 0% ($n = 9$) and 103 4 ± 0 6% ($n = 9$), respectively The intra-day reproducibility analysis of samples of serum, liver or kidney homogenate spiked with styrene (0 1—180 0 μg/ml) gave a mean C V of 3.8 ± 0 8% ($n = 20$), 4 1 ± 0.4% ($n = 20$) and 4.6 ± 0.6% ($n = 20$), respectively The inter-day reproducibility of the analysis of styrene-spiked sera, liver or kidney homogenate (0 1—36.0 μg/ml) lead to a mean C V of 4 3 ± 1 1% ($n = 6$), 5.6 ±

1.0% (n = 6) and 5.3 ± 0.6% (n = 6), respectively Accuracy determination of this method indicated a mean deviation of 5 1 ± 0.5% (n = 6), 6.0 ± 0.4% (n = 6) and 5.5 ± 0 5% (n = 6), in styrene-spiked samples of serum and liver or kidney homogenate (0 1—90 0 μg/ml), respectively.

Finally, we have tested a possible interference of the polar metabolites of styrene, namely hippuric, mandelic and phenylglyoxylic acids (which are found in greater amounts in urine) with the analysis of styrene itself The peaks of these metabolites were found to show up much earlier (mean retention time = 1 min) than those of styrene and benzene.

CONCLUSION

This method is found to have an excellent accuracy, sensitivity and precision, and thus offers a great analytical potential for measuring styrene in various tissues of animals. Its rapidity, simplicity and low cost make it very suitable for use with any HPLC instrument

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