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A Novel Approach Based on Solid Phase Microextraction Gas Chromatography and Mass Spectrometry to the Determination of Highly Reactive Organic Compounds in Cells Cultures: Styrene Oxide

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A solid phase microextraction (SPME) gas chromatography/mass spectrometry (GC/MS) method was developed to assess actual doses of highly reactive organic compounds like styrene oxide (SO) in exposed cell cultures. Using SPME, we set up a method to measure accurately extracellular SO concentrations as well as to obtain an approximate assessment of intracellular levels. The SPME−GC/MS method was developed and validated using two different coating materials, carboxen-PDMS and polyacrylate. In cell-free systems, linearity was established over 3 orders of magnitude for both fibers, but carboxen-PDMS showed higher extraction efficiency and a lower limit of detection (0.5×10^{-7} vs 10^{-6} M for polyacrylate). Precision calculated as % RSD was within 4−16% for all intra- and interday determinations. Experiments performed to study SO stability in cell-free medium showed a time-dependent decrease in SO concentration (11% of initial the concentration after 24 h), mostly due to the spontaneous hydrolysis of SO into styrene glycol, which was measured by liquid chromatography/tandem mass spectrometry (LC/MS/MS). When the neuronal cell line (SK-N-MC) was exposed to a nominal concentration of 0.3×10^{-4} M SO, the actual concentration measured in the supernatant was considerably lower and was found to decrease during incubation. Intracellular SO was estimated indirectly, by difference between the amount measured in the medium without cells and in the supernatant of the cell-containing medium.

Introduction

Over the past decades, an increasing number of test systems have been developed for evaluating toxic properties of chemical compounds. Many such systems do not rely on the use of intact animals but make use of biological systems with a lower level of organization than the organism: isolated organs, cell cultures, and subcellular systems. These in vitro systems have been extremely useful in studying the molecular basis of a chemical's biological activity, including its mechanism(s) of toxic action (*1*, *2*). In toxicology, as well as in pharmacology, the dose to which target cells are exposed to is usually expressed in terms of nominal concentrations, which are used to calculate the median effective concentration (EC_{50}). The main disadvantage of this approach is that the actual dose of volatile organic compounds delivered to target cells is not adequately assessed. Not only the volatility but also the solubility of tested compounds in the medium as well as their chemical reactivity may considerably affect their bioavailability. This can have important implications for a quantitative description of dose−response relationships. Indeed, control over exposure conditions and the accurate estimation of actual doses represent two major issues in the use of cells to assess the toxicity of volatile chemicals.

7,8-Styrene oxide (SO) is an industrial chemical used in the manufacture of epoxy resins; as an intermediate in the preparation of various agricultural chemicals, cosmetics, and plastics; and in the processing of textiles and fibers. SO is also the main electrophilic intermediate metabolite of styrene, a chemical used in several industrial applications, e.g., production of styrene monomer, polyester resins, synthetic rubber, and in the manufacture of glass fiber reinforced plastics. Exposure to styrene and SO is correlated; more than 80% of inhaled styrene is converted by cytochrome P450 to SO (*3*). As an epoxide, SO is able to undergo reactions with various nucleophilic groups in tissue components, causing effects such as growth inhibition, cytotoxicity, and mutagenicity (*4*). SO is responsible for several single strand breaks to occur (*5*). SO is a proven animal carcinogen and is classified as a possible human carcinogen (group 2B) by IARC (*6*). Styrene and SO have been shown to have different toxic effects on the central nervous system in animals (*7*) as well as in humans (*8*).

Several analytical methods for the determination of SO in biological matrixes and in tissues are reported in the literature. They are mainly based on the liquid−liquid

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extraction (LLE) of the analyte with *n*-pentane (*9*), *n*-hexane (*10*−*12*), and benzene (*13*), followed by analysis using gas chromatography/mass spectrometry (GC/MS) or GC with other detectors. Although high extraction efficiencies are reported, LLE is time-consuming and laborious. Moreover, sample handling may increase the risk of introducing experimental errors. Most of these problems can be overcome using solvent-free extraction techniques, like solid phase microextraction (SPME). SPME has gained a lot of interest in a broad field of applications, including food (*14*), environmental (*15*), biomedical (*16*), and pharmaceutical analysis (*17*). By contrast, the application of SPME to in vitro systems is limited to a single example (*18*).

In a recent paper, we have demonstrated that human neuroblastoma cells (SK-N-MC) exposed to nominal concentrations of SO ranging from 0.05 to 1 mM die through apoptotic pathways (*19*). Preliminary results indicated that actual doses were much lower than nominal levels. SO concentrations measured in the medium decreased over time; the total dose in the 24 h was 36% of the administered one. Here, we present a novel approach based on SPME−GC/MS to measure accurately extracellular SO concentrations as well as to obtain an approximate assessment of intracellular levels, with the aim of obtaining a more accurate estimate of the actual SO dose involved in neuronal cell death.

Experimental Section

Chemicals. Racemic SO (97% pure), styrene glycol (SG, 97%), styrene-d_8 (98%) used as GC internal standard (IS), DMSO (99.9%), and Dulbecco's modified medium (DMEM) were obtained from Sigma-Aldrich (Milan, Italy). Because of its high reactivity, SO standard stock solutions (30%) were prepared in DMSO de novo for each experiment, while SG (4×10^{-5} M in acetonitrile) and IS (10^{-3} M in methanol) stock solutions were stored up to 1 month. HPLC grade methanol, acetonitrile, and water were from Lab-Scan (Dublin, Ireland).

Sample Preparation to Study Equilibration Time and Partition Coefficients. Experiments were carried out in airtight vials (20 mL) keeping the same medium/air volume ratio of the flasks used in in vitro experiments. Equilibration time and medium/air partition coefficients ($K_{m/a}$) were estimated at three different SO concentrations (10^{-5}, 3×10^{-4}, and 10^{-3} M, respectively) maintaining either a 0.4 or a 0.04 SO/DMSO ratio. All experiments were carried out using racemic SO. After SO had reached the equilibrium between medium and air at 37 °C, $K_{m/a}$ was calculated by direct splitless injections of known volumes of headspace of standard samples and thus analyzed by GC/MS. The amounts injected were calculated by linear equations obtained by direct splitless injection of standard solutions.

Preparation and Exposure of Culture to SO. Human neuroblastoma cells (SK-N-MC) were grown in DMEM supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. For the experiments, cells were plated in a 25 mL flask (250 000 cells/mL). After 24 h, cells were treated with 3×10^{-4} M racemic SO. Because SO is not soluble directly in the aqueous growth medium, DMSO was used as the solubility vehicle, at a final concentration of 0.05% or less (concentration which is known not to be lethal for cells). Calibrating solutions were prepared in medium by diluting the SO stock solution prepared in DMSO. Control cultures were treated with volumes of medium containing DMSO dilutions equivalent to those in treated cultures (*20*). After 0, 8, 16, and 24 h of incubation, cell viability was assessed as described by Darè et al. (*19*), and 1 mL of supernatant was placed in 4 mL SPME vials to measure SO concentration in medium by SPME−GC/MS. For each exposure time, samples were collected in triplicate. An aliquot was stored for extraction with *n*-pentane. Styrene-d_8 was added to samples as IS (2 μL of the stock solution, final concentration 2×10^{-6} M). An additional aliquot was stored for the determination of SG by LC/MS/MS. Thus, samples were stored at −80 °C until analysis.

Sample Preparation to Study SO Stability. All of the experiments previously reported were repeated in the absence of cells to evaluate SO stability in the culture medium. The effect of different storage conditions (addition of salt, temperature, and time of storage) on SO degradation was then investigated as follows: several culture medium samples (1 mL) containing 3×10^{-4} M SO were prepared both without and with salt (0.5 g/mL) and were stored both at −80 and 37 °C, for different time periods, i.e., 3 days and 16 h.

SPME Procedure. Sampling by SPME was carried out using a 75 μm carboxen-PDMS and 85 μm polyacrylate fiber (Supelco, Bellefonte, PA). The fiber was exposed in the headspace of samples for 20 min at 80 °C under stirring. After the sampling, the fiber was immediately desorbed into the GC injection port at 280 °C for 10 min and no carryover was observed. The sampling was performed using a Combi/Pal System auto sampler (CTC Analytics, Zwingen, Switzerland).

Extraction by *n*-Pentane. Medium samples (1 mL) collected at different incubation times were transferred in 4 mL SPME vials containing 2 mL of *n*-pentane and 2 μL of IS 10^{-3} M solution. The vials were sealed with PTFE-lined screw caps and were vortexed for 30 s. The phases were then separated by centrifugation at 2000*g* for 5 min, and the organic phase extract was concentrated to about 25 μL under a gentle stream of nitrogen. Thus, 1 μL was analyzed by GC/MS (*9*).

GC/MS. The analyses were carried out on a HP 6890 GC equipped with a 5973 mass spectrometer (Hewlett-Packard, Palo Alto, CA). Separation was performed on a CP-SIL-5CB-MS column (30 m × 0.20 mm i.d., 0.25 μm film, Chrompack, Middelburg, The Netherlands). The temperature program was as follows: 100 °C hold for 5 min, rate 10 °C/min to 120 °C, hold for 2 min. Hydrogen was used as the carrier gas (1 mL/min). The chromatographic run was complete in about 9 min. The acquisition was performed in selected ion monitoring (SIM) mode, by acquiring the signals of the following ions *m*/*z* (dwell time in parentheses): 89 (60), 90 (70), 91 (30), and 120 (90) for SO and 82 (120), 84 (60), 110 (60), and 112 (30) for IS. A solvent delay of 1.5 min was necessary to protect the filament from the solvent vapors. Calibration samples were prepared in the medium in the 0.5×10^{-6} to 3×10^{-3} M concentration range.

LC/MS/MS. LC/MS/MS analyses were carried out using a Perkin-Elmer series 200 liquid chromatograph coupled with a PE-Sciex API 365 (SCIEX, Concord, Canada) triple-quadrupole mass spectrometer equipped with an ionspray interface for pneumatically assisted electrospray ionization. Chromatography was obtained on a Supelcosil LC-18-DB column (75 mm × 3 mm i.d., 3 μm, Supelco) with a mobile phase made of water and acetonitrile (75/25, v/v). SG was ionized in the positive ion mode (IS voltage, 6000 V; orifice voltage, 20 V). Acquisition was performed in selected reaction monitoring mode by following the transition characteristic of SG, *m*/*z* 121→103 (collision energy, 17 eV). Calibration samples were prepared in the medium in the 3×10^{-6} to 3×10^{-4} M concentration range ($y = bx$, $b = 91.790$, $r^2 = 0.998$, limit of detection (LOD) = 10^{-6} M). Before injection, samples were ultrafiltered on Centricon YM-10 (Millipore, Bedford, MA).

Statistics. Statistical analysis was carried out using the SPSS/PC+ software (Version 10.0 for Windows, Chicago, IL).

Results

Equilibration Time and Partition Coefficients. Experiments aimed at determining the time needed by SO to reach the equilibrium between medium and headspace at 37 °C were carried out in a closed system (airtight vials), where the medium/headspace volume ratio was chosen equal to that of the flasks used in in

Table 1. Medium/Air Partition Coefficients ($K_{m/a}$) of SO Calculated at Two Different SO/DMSO Ratio Values Both with and without Serum[a]

	medium (+ serum)	medium (− serum)
$K_{m/a}$ (SO/DMSO = 0.4)[b]	1060 ± 60	981 ± 166
$K_{m/a}$ (SO/DMSO = 0.04)	1520 ± 18	1078 ± 159

[a] Mean values for nine replicates, obtained at three different concentration levels (10^{-5}, 3×10^{-4}, and 10^{-3} M). [b] Operative conditions reported in the literature.

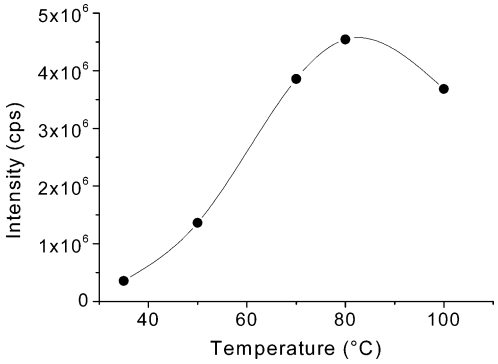

Figure 1. Temperature-dependent amount of SO extracted for a 20 min sampling using a 0.75 μm Carboxen-PDMS fiber. Each determination was performed in triplicate.

vitro studies. Equilibrium in static conditions was reached in about 10 min. Medium/air partition coefficients ($K_{m/a}$) were calculated both with and without serum to evaluate SO solubility in the medium at various concentrations (10^{-5}, 3×10^{-4}, and 10^{-3} M), maintaining either a 0.4 or a 0.04 SO/DMSO ratio. $K_{m/a}$ was not influenced by SO concentration (not shown), but it was higher in the presence of serum and with higher DMSO concentrations (SO/DMSO = 0.04), according to the hydrophobic characteristics of SO (Table 1).

SPME Method Development: Sampling Time, Extraction Efficiency, and Salting-Out Effect. Sampling at 80 °C under stirring conditions, SO was found to reach the equilibrium among the three phases, i.e., the medium (the matrix), the headspace, and the fiber, in 20 min using both coating materials. At lower temperatures, longer equilibrium times were observed. Figure 1 shows the temperature-dependent amount of SO extracted by carboxen-PDMS for a 20 min sampling. Extraction efficiency was calculated from the comparison of the areas obtained through direct splitless injection of known amounts of SO and the results of the SPME analysis of medium samples containing the same amount of SO. This experiment was carried out in both the presence of salt (0.5 g/mL) and without salt, at three different SO concentration levels (10^{-5}, 3×10^{-4}, and 10^{-3}M). The intermediate 3×10^{-4} M level was studied because this is the SO concentration reported in the literature for most in vitro experiments, and the highest one was chosen to exclude saturation of the SPME fiber. Because similar results were obtained for all tested concentrations, mean values of all determinations are reported in Table 2. Extraction efficiency was compared to that obtained with *n*-pentane. A negative salting-out effect on the percentage of SO extracted was observed.

Validation of SPME−GC/MS Procedure. The method was validated for both the coating materials by studying linear dynamic range, LOD, and analytical precision, calculated as % RSD for all intraday and interday determinations. The results are summarized in Table 3 for both fibers. A wide linear dynamic range, an imprecision lower than 16% for all determinations, and lower LODs (0.5×10^{-7} M for carboxen-PDMS) make this method appropriate to monitor SO concentrations during in vitro experiments.

Table 2. Amount (%) of SO Extracted at the Equilibrium by LLE (*n*-Pentane) and by Two Different SPME Fibers[a]

carboxen-PDMS		polyacrylate		*n*-pentane[b]
10.3 ± 0.9 (+ NaCl)	15.2 ± 1.2 (− NaCl)	1.3 ± 0.05 (+ NaCl)	2.1 ± 0.4 (− NaCl)	11.9 ± 2.1

[a] Extraction efficiency was calculated at three concentration levels (10^{-5}, 3×10^{-4}, and 10^{-3} M), and mean values for nine replicates are reported. [b] Extraction with *n*-pentane was chosen as reference from the literature (*9*).

Table 3. Validation of the SPME−GC/MS Method: Linear Range, Correlation Coefficients, Limits of Detection, and Intra- and Interday Precision

	carboxen-PDMS	polyacrylate
linear range (M)	0.5×10^{-6} to 10^{-3}	10^{-6} to 10^{-3}
b[a]	0.041 ± 0.002	0.036 ± 0.001
r^2	0.998	0.988
LODs (M)[b]	0.5×10^{-7}	10^{-6}
precision (% RSD)[c]		
intraday (%)	4.3−8	4−15.7
interday (%)	6.2−9.0	5.9−13.4

[a] Calibration fitting: $y = bx$ ($n = 18$); ± values are confidence intervals for the 95% probability level. Linear regression analysis was carried out using the least-squares method. [b] Limit of detection (S/N = 3) calculated under SIM conditions. [c] Intra- and interday precision ($n = 9$) were calculated at three concentration levels: 10^{-5}, 3×10^{-4}, and 10^{-3} M.

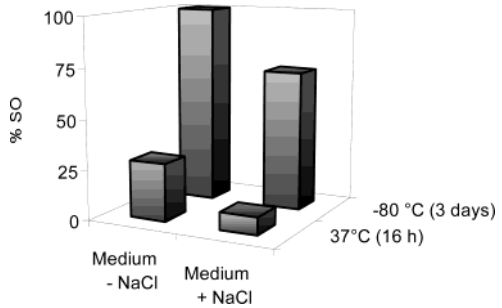

Figure 2. Effect of several factors (temperature, time of storage, and addition of salt) on SO degradation in culture medium. Experiments were carried out in medium without cells and in airtight vials to avoid evaporation.

Determination of SO Stability. Experiments aimed at establishing suitable sample storage conditions showed no degradation of the analyte when samples were stored at −80 °C for 3 days (residual SO, 96 ± 9.5%), while storage at 37 °C produced a detrimental effect on SO concentrations (28.8 ± 9.6%), as displayed in Figure 2. Experiments repeated in the presence of salt showed a higher SO degradation, even more evident at 37 °C. For these reasons, all samples were collected without salt and stored at −80 °C until analysis. Because in vitro experiments require 37 °C, additional experiments to evaluate SO stability were performed in culture medium at working temperatures in the absence of cells. SPME determination of SO concentration at different sampling times showed a time-dependent depletion of the analyte. This behavior is partly due to evaporation of SO and partly to its transformation into SG, as demonstrated by LC/MS/MS analysis on the same medium samples.

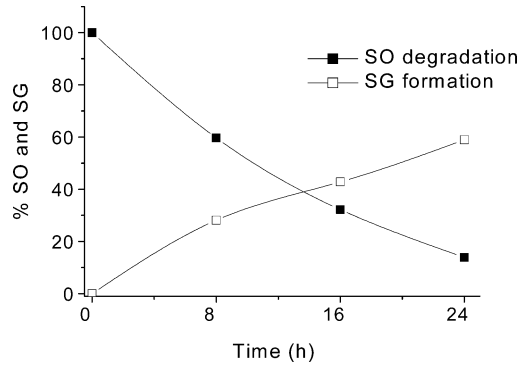

Figure 3. Time-dependent percent hydrolysis of SO into SG. After 24 h, about 60% of SO was degraded into SG. Experiments were performed in cell-free medium at 37 °C. SO and SG were measured by SPME−GC/MS and LC/MS/MS, respectively.

Figure 3 shows the proportional increase in SG concentration, corresponding to the decrease of SO.

Application: Determination of Actual SO Concentration in in Vitro Experiments. The SPME−GC/MS method was applied to the determination of SO extracellular concentration in SK-N-MC cells treated with 3×10^{-4} M SO (SO nominal concentration) and incubated for 8, 16, and 24 h. Cell viability assessed using Trypan Blue staining (Figure 4a) showed a time-dependent cytotoxicity, whereas no changes were observed in control cultures. At each incubation time, SO was measured both in cell-free culture medium (SO_m) and in cell-containing medium (SO_s), the degradation into SG being assessed in parallel (Figure 4b). Both SO_m and SO_s showed a significant time-dependent decrease as compared to the initial dose. In samples treated with SO, an increase in SG was also evident, both in cell-containing medium (SG_s) and in cell-free samples (SG_m), with a parallel trend between 8 and 16 h, the formation of SG being lower in the presence of cells. In this interval, the SO absorbed by cells at each incubation time was estimated indirectly by difference of the experimental points ($SO_m - SO_s$). At each time point, extrapolated values were related to the number of viable cells (Figure 4c) and values obtained were expressed as fmol of SO per cell. These results indicated that SO uptake is maximum between 0 and 8 h. At 16 and 24 h, SO concentration was significantly decreased as compared to the 8 h of incubation.

Discussion

SPME is a solvent-free sampling technique, which has deeply modified the way of introducing samples in GC. Among the number of advantages recognized to SPME, such as low cost, no carryover effects, full automation, excellent precision, and wide linear range, the applicability to complex matrixes together with the possibility of handling small sample volumes make this technique suitable for sampling organic chemicals from in vitro systems relying on tissues and cells. Nevertheless, SPME has not been extensively applied to this field. In the only previous study dealing with the determination of toxicologically relevant concentrations in vitro, SPME was used in a negligible depletion mode, i.e., extracting only small amounts of analytes from the sample, and differences were reported between nominal and bioavailable concentrations; these differences were higher for more hydrophobic compounds (*18*). In the present study, the

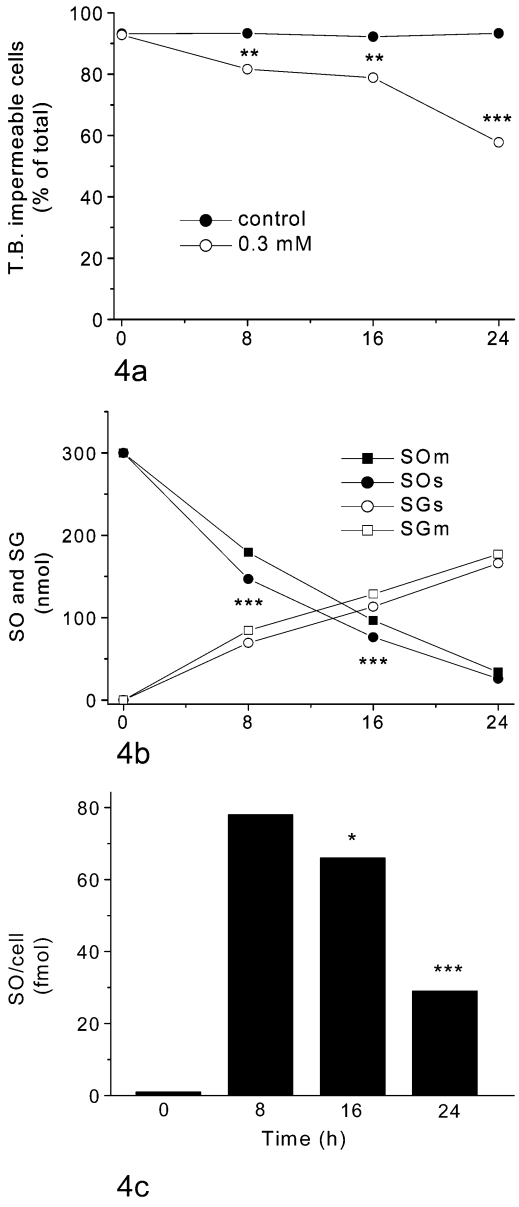

Figure 4. (a) SO-induced alterations of membrane permeability. SK-N-MC cells exposed to SO were detached from the surface, and then, aliquots of the cell suspension were stained with Trypan Blue and counted at the microscope. Values are means ± SEM of three independent determinations. Statistical analysis of the values obtained at each time point was performed with ANOVA test. **Significantly different from control ($p < 0.01$). ***Significantly different from control ($p < 0.001$). (b) SO and SG measured in cell-free culture medium (SO_m and SG_m) and in cell-containing medium (SO_s and SG_s). Aliquots of sample incubated with SO in the presence or absence of cells were collected to measure SO and SG by SPME−GC/MS and LC/MS/MS, respectively. Values are means ± SEM of three independent determinations. Differences between SO in medium (SO_m) and supernatant (SO_s) were assessed using a two-way ANOVA model. Differences at each sampling time were assessed using the Student's *t*-test for independent samples. ***Significantly different from SO_m ($p < 0.001$). (c) Femtomoles of SO per cell. SO inside the cells was estimated indirectly by difference ($SO_m - SO_s$). At each time point, the extrapolated values were related to the number of viable cells. Differences at each sampling time were assessed using the Student's *t*-test for independent samples. *Significantly different from fmol of SO per cell at 8 h ($p < 0.05$). ***Significantly different from fmol of SO per cell at 8 h ($p < 0.001$).

SPME technique was used in a different way, i.e., extracting the highest amount of SO from culture me-

dium at the equilibrium, with the aim of monitoring the analyte concentration over the whole incubation period during in vitro experiments.

The SPME method development was rigorously set up to identify the best operative conditions and to obtain the highest sensitivity for the analyte under investigation. Between the two different fiber materials tested and compared in this study, carboxen-PDMS showed a better affinity for SO, a wider linear dynamic range, a better precision, and a lower LOD (Table 3). Because the partition coefficients of analytes are partially determined by the interaction between target analytes and the matrix, the nature of the matrix can be modified by changing several physical and chemical parameters, such as the temperature, the pH, and the ionic strength, with the aim of influencing the coating/matrix partition coefficients of the analytes. Critical issues in method optimization were the sampling temperature and the addition of salt. A temperature of 80 °C hold for 20 min was found as the optimum condition for SO, a hydrophobic compound with a high boiling temperature (194 °C). At higher temperatures, the amount of SO extracted was found to decrease, as shown in Figure 1. Although heating tends to drive analytes out of the liquid phase and into the headspace, heating also alters the partitioning of the analyte between the headspace and the fiber to favor the headspace. In general, the addition of a salt (e.g., NaCl or Na_2SO_4) to aqueous samples is frequently used to drive polar compounds into the headspace (*21*), but it has a relatively insignificant effect on nonpolar compounds such as SO. Experiments performed to study the salting-out effect showed that SO extraction decreased in the presence of NaCl as shown in Table 2. This effect could not be ascribed to the formation of clots or precipitation of substances normally present in medium (e.g., proteins); the solution was clear during sampling, despite the addition of salt. The lower yield of SO extraction was due to the spontaneous degradation of SO during sampling time (20 min). In fact, additional experiments carried out to evaluate SO stability showed a higher loss in analyte concentration when the medium was stored, in the presence of salt, at 37 °C for several hours before analysis (Figure 2). This effect was probably due to spontaneous hydrolysis of SO to SG; such a hydrolysis is favored in either slightly acidic or basic conditions, like in culture medium systems (*22*). This hypothesis was confirmed by monitoring both SO (by SPME−GC/MS) and SG (by LC/MS/MS) in medium samples collected at different incubation times. Increases in SG concentrations were found to correspond to proportional decreases in SO concentrations, as shown in Figure 3. Hence, even if the presence of salt could increase to a certain extent SO headspace concentration, on the other hand, it was found mainly to promote the spontaneous hydrolysis of SO into SG.

Another critical step in method development was the solubilization of SO in the medium and the preparation of a SO solution suitable for in vitro experiments. Working SO solutions are usually prepared in medium starting from a stock solution in DMSO. In fact, SO is not soluble directly in the aqueous medium, except at very low concentrations. Because of its high affinity toward the medium, DMSO works as a solubility vehicle for SO, increasing its concentration in aqueous environments. Experiments conducted to assess whether the relative SO/DMSO ratio could influence the solubility of SO in medium showed that the partition coefficient medium/air obtained under the same conditions reported in most published papers (i.e., with a SO/DMSO ratio of 0.4) is considerably lower (1060 ± 63) than the partition coefficient (1520 ± 18) measured using a 10-fold higher concentration of DMSO (SO/DMSO = 0.04). These data clearly indicate that the retention capacity of the solvent interferes with SO partition coefficients.

Application to Cell Cultures Exposed to SO. The first step in the major metabolic pathway of styrene is formation of SO. This fairly reactive metabolite binds covalently to macromolecules (*6*). SO is further metabolized by hydrolysis to SG or by conjugation with glutathione to produce mercapturic acids. SG is then oxidized to mandelic acid (MA) and phenylglyoxilic acid (PGA), the main urinary metabolites of styrene in man. Different neurotoxic effects in animals (*7*) and in humans (*8*) have been associated to both styrene and SO exposure. Human neuroblastoma cells (SK-N-MC) exposed to nominal concentrations of SO ranging from 0.05 to 1 mM die through an apoptotic pathway. Preliminary results indicated that actual doses were much lower than nominal levels (*19*). Here, we present a novel approach based on SPME-GC/MS and LC/MS/MS to determination of SO and SG in culture medium, having excluded the formation of both mercapturic acids and MA and PGA (data not shown), with the aim of obtaining a more accurate estimate of the amount of SO involved in neuronal cell death.

A decrease in cell viability was observed between 0 and 8 h, when living cells were 80% as compared to controls. At 16 h, a similar proportion of viable cells was observed, such a proportion being reduced to 60% at 24 h (Figure 4a). Although SPME−GC/MS is a suitable technique for the measurement of free SO in the medium of cell cultures, it cannot detect SO taken up by cells directly and presumably bound to intracellular macromolecules, the determination of which would require the use of radioactivity measurements. Because radiolabeled standards are neither commercially available nor easy to obtain by synthesis, we tried to indirectly estimate the amount of SO absorbed by cells during the 24 h of incubation. The amount of absorbed SO was then extrapolated by dividing the differences between the areas under the curves in Figure 4b indicating the time-dependent decrease of SO without and with cells by the area under the curve indicating the time-dependent decrease of SO in the absence of cells, assumed as 100%. These data seem to show that about 14% of the available SO was taken up by cells. To support this hypothesis, a complementary decrease in the % amount of extracellular SG (13.5%), extrapolated in the same way, was observed in the presence of cells, indicating an actual decrease in the concentration of SO available in the medium. Because SG toxicity to SK-N-MC cells in separate experiments has been excluded (data not shown), we can infer that despite the fact that the absorbed SO is a small part of the total amount to which cells have been exposed, this concentration is sufficient to induce apoptotic cell death. After 16 and 24 h of incubation, a significant decrease in SO concentration was extrapolated as compared to the 8 h of incubation (Figure 4c). At 24 h, SO_m and SO_s were similar (Figure 4b) and cells were either at the equilibrium or achieved a saturation state, in any case being unable to absorb more SO. Another hypothesis is that the intracellular SO is totally bound to macromolecules,

although further investigations based on the use of radiolabeled SO are needed to test this hypothesis and to strengthen the validity of our results.

Conclusion

Although SPME−GC/MS is a very sensitive technique satisfying the requirements of linearity, accuracy, and precision, it cannot be considered as an alternative to reference techniques based on radiolabeled compounds. Nonetheless, we have demonstrated that it is suitable for the determination of the target dose of SO to cells cultures. We reported an application in which an in vitro model (neuronal cells) has been used to measure the amount of SO able to induce apoptotic cell death. The critical levels that we recorded are consistent with the hypothesis of styrene-induced neurotoxic effects after long-term low level exposure in vivo both in occupationally exposed workers and in experimental animals. Our data indicate that actual doses are much lower than nominal levels and decreased progressively with time mostly to the spontaneous hydrolysis of SO into SG. In conclusion, when using in vitro models to assess the toxicity of volatile substances, nominal concentrations provide only a rough estimate of the actual dose, which should be determined in each experimental situation. To our knowledge, this is one of the first studies focused on the evaluation of actual concentrations of a volatile and reactive substance, like SO, delivered to cell culture that makes use of new techniques (SPME−GC/MS and LC/MS/MS).

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