Solid-Phase Microextraction: Investigation of the Metabolism of Substances that May be Abused by Inhalation

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Abstract

Purified liquefied petroleum gas (LPG), a mixture of butane, isobutane, and propane, is commonly abused by inhalation. Little is known about the mammalian metabolism of these substances. Metabolism of other hydrocarbons, including *n*-hexane and cyclohexane, has been studied in vitro using a range of liver preparations, with metabolites analyzed by static headspace techniques. Solid-phase microextraction (SPME) for sampling metabolites in the headspace of incubates of volatile compounds with activated rat liver microsomes is investigated. Cyclohexanol and cyclohexanone were formed from cyclohexane and 1-, 2-, and 3-hexanol and 2-hexanone from *n*-hexane as predicted. Secondary alcohols are found for the other compounds studied, except for propene and isobutane, together with 2-propanone and 2-butanone from propane and *n*-butane, respectively. Samples from three individuals who died following LPG abuse contained a range of putative *n*-butane metabolites: *n*-butanol, 2-butanol, 2,3-butanediol, 3-hydroxy-2-butanone, and 2,3-butanedione. To our knowledge, the last three compounds have not been proposed as metabolites of *n*-butane in man. These might be produced through similar metabolic pathways to those of *n*-hexane and *n*-heptane. The findings indicate the value of SPME for investigating the metabolism of volatile substances and for detecting and monitoring exposure to these compounds.

Introduction

Deliberate inhalation of volatile substances in order to achieve intoxication [inhalant abuse, solvent abuse, "glue sniffing", and volatile substance abuse (VSA)] is a worldwide problem. Sequelae may include criminal or antisocial behavior, accidental injury while intoxicated, and sudden death (1–3). There have been at least 2104 sudden VSA-related deaths in the UK alone between 1971 and 2003 (4). Approximately 70% of deaths occur under the age of 20 (3). The toxicity of volatile substances depends on the compound and on the magnitude, duration, and route of exposure (3). Irreversible neurological or organ damage may be a long-term consequence of the abuse of toluene and possibly of other compounds (1,3,5). This topic is also of current interest in occupational toxicology (6). Substances abused include liquefied petroleum gas (LPG) (in fuel gases or cigarette lighter refills), aerosol propellants, solvents from adhesives, chlorinated solvents, and nitrous oxide (Table I). Purified LPG, used as cigarette lighter fuel, contains mainly propane, butane, and isobutane. Some unpurified LPGs, intended for direct use as fuels, contain up to 40% (v/v) of unsaturated hydrocarbons (mainly propene and butenes).

Many abused VSAs have short plasma half-lives (e.g., butanone, 0.5 h; dichoromethane, 0.7 h; and *n*-hexane, 1–2 h) (2,5). Data are lacking for the very volatile (C_3–C_5) hydrocarbons. These compounds may be readily lost from biological samples after collection if appropriate precautions are not taken (2,3). Hence, analytical confirmation of recent exposure can be problematic. With some compounds, the presence of polar, less-volatile metabolites with longer half-lives extends the detection window. Some examples are 2,5-hexanedione and 4,5-dihydroxy-2-hexanone, used as markers for the exposure to hexane and 2-hexanone [methyl butyl ketone (MBK)] (7,8). Others are hippuric acid for toluene and trichloroacetic acid for trichloroethylene exposure (2). However, little is known about the metabolism of the short-chain saturated (propane, butane, and isobutane) and unsaturated (propene, butene, and isobutene) hydrocarbons other than the suggestion that secondary alcohols and, where appropriate, the corresponding ketones may arise from the metabolism of the saturated compounds (8). *n*-Hexane metabolism, in contrast, has been studied in vivo in animals and in vitro in man (5). In vitro, *n*-hexane has been incubated with cytochrome P-450 enzymes and microsomal preparations with a nicotinamide adenine dinucleotide phosphate (NADPH)-generating system and either liver slices or supernatant (S9 fraction)

of centrifuged liver homogenates, along with the volatile metabolites produced, were analyzed in the headspace (HS) or after solvent extraction (5,9,10).

Solid-phase microextraction (SPME) (13) offers considerable potential to expand these studies. The use of SPME in the analysis of biological fluids for diagnostic purposes (14,15) and in the analysis of solvents and anaesthetic gases (3) has been documented. Volatile compounds with a wide range of polarities may be sampled directly from the HS, and semivolatile compounds can be extracted from biological fluids or incubation media by immersing the SPME fiber in the sample.

A preliminary study of the use of rat liver microsomes to investigate the metabolism of the components of unpurified LPG is reported. We set up and optimized incubation, sampling, and gas chromatographic (GC) conditions. The procedures were validated using *n*-hexane and cyclohexane, which are liquids at room temperature and, hence, easier to handle. They also have a known metabolism. The HS–SPME method was also applied to the analysis of autopsy samples from three individuals who were thought to have died as a result of VSA.

Experimental

Chemicals, reagents, and standards

Glucose-6-phosphate dehydrogenase type VII from baker's yeast, glucose-6-phosphate disodium salt hydrate (98–100%), magnesium chloride hexahydrate (99%), sodium chloride (99.5%), potassium carbonate (99%), anhydrous monobasic potassium phosphate (99+%), anhydrous dipotassium hydrogen orthophosphate (99%), potassium chloride (99.5%), and β-nicotinamide adenine dinucleotide phosphate (NADP) sodium salt were all from Sigma (Poole, UK). All analytical-grade solvents, reagents, and high-purity chemical standards (propane, *n*-butane, *n*-hexane, their possible mammalian metabolites, butene, *trans*-2-butene, *cis*-2-butene, isobutane, and propene) were from Aldrich (Poole, UK). Water was purified by reverse osmosis.

Standard solutions were prepared at an appropriate concentration by dissolving the analyte(s) in methanol (100 µL) and then diluting them with water to 250 mL. Standards supplied as gases were added individually to a clean 125-mL gas sampling bulb (Supelco, Poole, UK) by purging the vessel (5 s). The bulb was sealed (atmospheric temperature and pressure) and samples were removed via the silicone rubber septum using a gas tight syringe (0.5 mL). After each test compound was sampled, the sampling bulb and syringe were thoroughly purged (30 s) with pure nitrogen.

HS, SPME, and gas chromatography–mass spectrometry equipment

Twenty-milliliter glass headspace vials, 20-mm silicone rubber septa, 20-mm aluminium sealing rings, 12-mm magnetic mixing bars, SPME manual holder, and SPME fibers with different phases to enable extraction via absorption or adsorption mechanisms (13) [100-µm thick polydimethylsiloxane (PDMS), 85-µm thick polyacrylate, 65-µm thick carbowax (CW)–divinylbenzene (DVB), 75-µm thick carboxen (CAR)–PDMS, 50-µm thick StableFlex design DVB– CAR–PDMS] were from Supelco. The SPME fibers were conditioned prior to use by heating in a split/splitless GC injection port in a stream of helium for 2 h at 300°C (polyacrylate, CAR–PDMS fibers) or 1 h at 250°C (PDMS, CW–DVB, and DVB–CAR–PDMS fibers).

Analyses were performed on a 5890 series II GC linked to a 5971A bench top quadrupole mass spectrometer (MS) with data analyzed by ChemStation software (Agilent, Bracknell, UK). Compounds were separated using a CW fused-silica capillary column (30 m × 0.25-mm i.d., 0.25-µm film) (Alltech, Carnforth, UK) with an oven temperature program of 40°C (5 min), then increased 10°C/min to 220°C (15 min). Helium was used as the carrier gas (flow rate 1 mL/min). The gas chromatography (GC)–MS interface temperature was held at 280°C, and the electron impact ionization potential was 70 eV (ionization current 350µA). Mass spectra were obtained in the full-scan mode (scan range 30–550 amu). Compounds were identified by comparison with pure reference standards and by use of the NIST 98 Mass Spectral Search Program (Gaithersburg, MD) and Wiley 275 Mass Spectral e-Library mass spectral databases (John Wiley & Sons, New York, NY).

Compounds extracted on the SPME fiber were thermally desorbed in a narrow bore (0.75-mm i.d.) SPME injection liner (Supelco) at 270°C for 5 min, with the injector in the splitless mode (split valve initially closed for 2 min, then purged

Table I. Examples of Commonly Abused Substances and Their Volatile Components

Primary substance abused	Example products	Volatile components
Fuel gases	Cigarette lighter refills Domestic gas (bottled) Blow-torch fuels	Purified LPG (butane, isobutane and propane), propenes, and butenes if unpurified LPG
Aerosol propellants	Hair spray Spray paint Air freshener	Purified LPG, dimethyl ether, or various fluorocarbons (or both)
Glues	Contact adhesives Model glue	Toluene, xylenes, hexane, ethyl acetate, and butanone
Dry cleaning solvents		Trichloroethylene
Anaesthetic gases		Isoflurane, nitrous oxide, sevoflurane
Miscellaneous	Petrol Paint thinners Brake cleaner	Butanone, hexane, toluene, and aliphatic and aromatic hydrocarbons

with carrier gas at a split ratio of 20:1). In order to prevent carryover, the SPME fiber was further desorbed at 270°C for 5 min in a separate GC injector between each analysis.

Preparation of rat liver microsomal suspensions

Rat liver microsomal suspensions were prepared using standard methods. Briefly, a known weight of frozen rat liver was defrosted (4°C) in homogenization buffer [0.01 mol/L phosphate buffer containing 1.15% (w/v) potassium chloride], chopped finely, and transferred to a homogenizer. Additional buffer was added (10 mL/g liver) and the tissue homogenized (30 s). The crude homogenate was then centrifuged (11,500 rpm for 20 min at 4°C). The supernatant was removed from the sedimented nuclei and mitochondria and recentrifuged (37,000 rpm for 75 min at 4°C). The supernatant was decanted, and the microsomal pellet suspended in 1 mL of 0.25 mol/L phosphate buffer (pH 7.25, containing 30% w/v glycerol) per gram of liver. Portions of the suspension (0.5 mL) were flash frozen (liquid nitrogen) and stored at –70°C.

NADPH-generating system

The NADPH-generating system was prepared daily and stored at 4°C. It contained: glucose-6-phosphate, 1 mmol/L (3.0 mg/mL); NADP, 5 mmol/L (3.8 mg/mL); glucose-6-phosphate dehydrogenase, 1 unit (1.7 µg/mL); and magnesium chloride, 2.5 mmol/L (0.5 mg/mL) dissolved in 0.2 mol/L potassium phosphate buffer (pH 7.4).

Incubation of volatile compounds with microsomes and SPME extraction (definitive procedure)

One milliliter of the NADPH-generating mixture, 2.8 mL potassium phosphate buffer (0.2 mol/L, pH 7.4), and 0.2 mL of thawed rat liver microsome suspension was added to 20-mL glass HS vials containing a magnetic stirring bar. After sealing the vials, test compounds were added using a gas-tight syringe. Vials were incubated for 2 h (37°C) in a reciprocating (100 rpm) water bath and then transferred to a stirrer (150 rpm) water bath maintained at 70°C. Volatile compounds were extracted (30 min) from the HS with an SPME device fitted with a CAR–PDMS fiber.

Procedural blanks (no NADPH-generating system) and control samples (NADPH-generating system without the addition of the test substance or no added microsomal suspension) were extracted and analyzed in parallel.

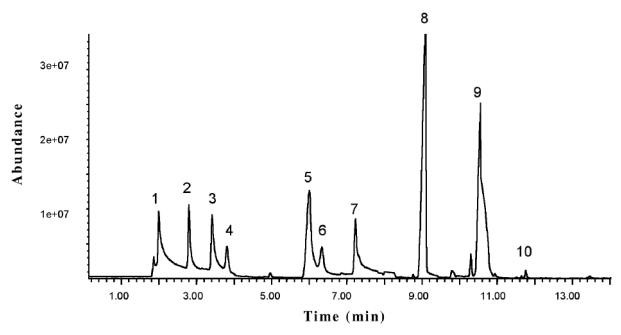

Figure 1. GC–MS analysis of an aqueous standard mixture of 11 known or predicted propane, *n*-butane, and *n*-hexane metabolites. HS extraction (75 µm thick CAR–PDMS, 70°C, 30 min). GC–MS conditions as described in Experimental section. Peak numbers [concentration in mixture (µmol/L) in brackets]: *n*-hexane (0.15), 1; 1,2-epoxybutane (0.23), 2; 2-butanone (0.22), 3; 2-propanol (2.61), 4; 2-butanol (1.09), 5; 1-propanol (2.68), 6; 2-hexanone (0.02), 7; 1-butanol (1.09), 8; 2-hexanol (0.16), 9; 3-hydroxy-2-butanone (0.45), 10; and 2,3-butanediol (0.44) was not detected.

Autopsy samples

Samples (whole blood, urine, adipose, brain, and lung tissues) collected into Sterilin containers at autopsy from three individuals thought to have died as a result of inhalation of purified LPG were analyzed to establish the cause of death. Four milliliters of whole blood or urine was added to a 20-mL HS vial containing a magnetic stirrer and sealed. Portions (2–8 g) of each tissue were chopped roughly while still frozen, added to a vial, and sealed immediately. Because of the limited availability of samples, only single analyses were performed. Volatile compounds were extracted (30 min, 50°C) from the HS using a CAR–PDMS SPME fiber and analyzed by GC–MS.

Results and Discussion

Optimization of GC conditions

Because the study was concerned with the identification of the semipolar and polar metabolites of volatile compounds, as well as the parent substances, a polar (CW) GC stationary phase was selected. This gave good resolution and peak shapes with minimal tailing. Most compounds of interest eluted within 15 min (Figure 1). Cryo-cooling of the GC oven was not used, nor were alternative thick-film GC stationary phases tested to attempt to separate the most volatile (C_3 and C_4) gases from the air peak.

Optimization of SPME extraction conditions

The type of SPME fiber and extraction conditions used have a significant effect on the range and amount of analytes extracted from the HS (13). Using different mixtures of aqueous standards, containing the known or postulated mammalian metabolites of propane, *n*-butane, cyclohexane, and *n*-hexane (concentration of each compound in the mixture being adjusted to give a suitable GC–MS response), the extraction efficiency of five different types of SPME fiber were investigated. The HS was sampled at 70°C (30 min). The CAR–PDMS SPME fiber was at least ten times more efficient than the other fibers studied in the extraction of the compounds tested. This was not surprising because the manufacturers suggest that the CAR–PDMS fiber (based on a porous carbon with a very high surface area) has been specifically designed to extract very low molecular mass compounds. This fiber was selected for subsequent work. The relatively polar compound, 2,3-butanediol, was not detectable with any fiber when present at 0.44 µmol/L in an aqueous solution.

To investigate the influence of sampling temperature, an aqueous mixture of the predicted *n*-butane and *n*-hexane metabolites was extracted over the temperature range 30–80°C. The results indicated that there was no optimal extraction temperature for this diverse range of compounds (Figure 2). The extraction of 2,5-hexanediol increased significantly at 50°C, but this may be because of the high melting point (~ 50°C) of this

compound. Although using a higher extraction temperature can increase the concentrations of volatile compounds in the HS, at higher temperatures, compounds extracted onto the SPME fiber can also be desorbed from the fiber back into the HS (13). Even when using an extraction temperature of 80°C, the hydrophilic compound, 2,3-butanediol, could not be detected at 0.44 µmol/L. As a compromise, 70°C was selected, particularly as the *n*-hexane metabolites were best extracted at this temperature. This temperature also denatures microsomal enzymes and thereby terminated the enzyme reactions at the end of the incubation period.

To investigate the effect of sampling time, the same mixture of compounds was extracted over periods of 5 to 70 min using a CAR–PDMS SPME fiber (70°C). No one extraction time was found to be optimal. Figure 3 shows that equilibrium was achieved rapidly (within 5 min) for the possible butane metabolites and by 30 min for 2-hexanol and 2-hexanone. Equilibrium was not attained for 2,5-hexanedione and 2,5-hexanediol, even after 70 min. As a compromise between efficiency and practical considerations, an extraction time of 30 min was selected.

To investigate the effect of adding a salt on the extraction (13) of the same aqueous mixture of predicted *n*-butane and *n*-hexane metabolites, sodium chloride or potassium carbonate was added in excess, and the HS was sampled for 30 min at 50°C. Neither salt significantly increased the extraction efficiency. It was considered undesirable to add salts to the microsomes in the vial (see later) at the start of incubation because of the risk of denaturing the enzymes. Adding salt after the incubation would necessitate opening the vial with the risk of losing volatile compounds. Salts were, therefore, not added to the incubation vials at any stage.

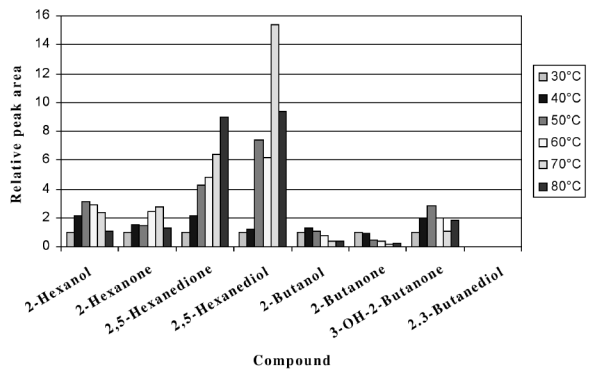

Figure 2. The influence of temperature on the extraction of known or postulated metabolites of *n*-hexane and *n*-butane. An aqueous mixed standard (4 mL) was placed in a HS vial and extracted (30 min) using a 75-µm thick CAR–PDMS fiber. The relative peak areas are normalized to those at 30°C.

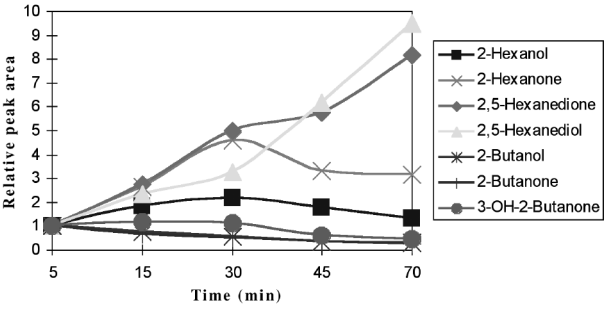

Figure 3. Effect of sampling time on the extraction of metabolites of *n*-hexane and putative metabolites of *n*-butane (single observations). Relative peak areas normalized to those at 5 min.

Optimization of microsomal incubations

The procedure reported by Mortensen et al. (11) was optimized for the in vitro metabolism of the test compounds (i.e., to gain the maximum conversion of parent compound to metabolites). The relative amounts of constituents used in NADPH-microsomal enzyme incubations can significantly affect the amounts of metabolite produced (16). Initial experiments were conducted using *n*-hexane (1 µL) as the substrate. Being a liquid at room temperature, *n*-hexane was easier to work with than volatile gases, which may be difficult to measure accurately and to dissolve in the aqueous matrix. Three different concentrations (1.5, 5, and 15 mmol/L) of NADP were tested with 0.2 mL of microsomal suspension. The results showed that increasing concentrations did not increase production of 1-hexanol, 2-hexanol, or 3-hexanol. However the yields of 2-hexanone increased with increasing amounts of NADP.

The volume of microsomal suspension added to the vial was varied (0.2, 0.8, and 2.0 mL) when using a fixed amount (5 mmol/L) of NADP. Unexpectedly, increasing the amount of suspension in the system did not increase production of 1-hexanol, 2-hexanol, 3-hexanol, or 2-hexanone. It may be that the NADPH-generating system could not produce enough co-factor within the vial to fully activate all the enzymes present. As a compromise, and taking into account the cost and availability of the materials, 5 mmol/L of NADP with 0.2 mL of microsomal suspension were selected for the study.

Microsomal incubations with volatile hydrocarbons

Using the definitive procedure, *n*-hexane (1 µL) and cyclohexane (1 µL) as well as saturated and unsaturated C_3 and C_4 hydrocarbons (1 and 5 mL of each gas) were incubated with the microsomal suspension NADPH-generating system. The metabolites produced were extracted from the HS using a CAR–PDMS fiber and identified by GC–MS. Reagent blanks and controls were analyzed in parallel.

Metabolites were detected for all of the compounds tested except isobutane and propene (Table II). Failure to detect any products from these gases may have been because a sufficient amount of substrate was not dissolved (low solubilty) in the incubation mixture, despite vigorous shaking (17). Further work is needed to increase the efficiency of the in vitro incubation system. The metabolites found for cyclohexane and hexane (Figure 4) are in accordance with the reported metabolism of these substances (2,5,18). In animals and man, *n*-hexane is oxidized to 1-, 2-, or 3-hexanol. 2-Hexanol then undergoes further oxidation to a range of compounds, including 2,5-hexanedione, which is neurotoxic (Figure 5A) (5,10).

In vitro studies with microsomal preparations, cytochrome P-450 enzymes, liver slices, and S9 fractions of liver homogenates indicate that oxidation of *n*-hexane to 1-, 2-, or 3-hexanol and 2,5-hexanediol is accomplished by microsomal cytochrome P-450 enzymes (5,9,11). At least four enzymes may be involved in the formation of the hexanols, and 2-hexanol is the

major metabolite. A different cytochrome P-450 enzyme converts 2-hexanol to 2,5-hexanediol (9). 2-Hexanol and 2,5-hexanediol are substrates of cytosol alcohol dehydrogenase (9,10). Studies with rat liver slices and S9 preparations show that both microsomal and cytosolic enzymes are involved in the metabolism of 2-hexanol to 2,5-hexanedione (5,11). Production of 2-hexanone by the microsomal preparation was, perhaps, surprising. However, the cytochrome P-450 complexes are known to oxidize an alcohol to an aldehyde, as in the case of the microsomal ethanol oxidizing system (19).

Alcohols were identified following incubation of all other compounds tested except propene and isobutane. There are no published observations for the formation of the 2- and 3-butene alcohols. 2-Butanol was the main metabolite formed from the incubation of *n*-butane with rat liver microsomes (20).

Analysis of *n*-butane metabolites in autopsy samples

Figure 6 shows the metabolites found in the HS of a fat sample (with no adjustment of pH) extracted (30 min at 50°C) using a CAR–PDMS SPME fiber. There was a peak of ethanol and a very large peak of 3-hydroxy-2-butanone (acetoin) with smaller peaks of *n*-butanol, 2-butanol, and 2,3-butanedione (diacetyl). 3-Hydroxy-2-butanone and a trace of 2,3-butanedione were found in blood, and a trace of 3-hydroxy-2-butanone was found in the liver. No putative *n*-butane metabolites were detected in the brain or urine. *n*-Butane, isobutane, and propane were not detectable in any of the tissues by our method but were found using conventional HS analysis on a long, thick-film, apolar GC column. Blood ethanol was 69 mg/100 mL.

Analyses of blood collected from the lung and brain from another individual who died of VSA contained large amounts of 2-butanone, together with smaller quanities of 2-butanol, 3-hydroxy-2-butanone, and isomers of 2,3-butanediol. Ethanol was not detected in his blood or urine. In contrast, in blood collected after death from a third victim of VSA, there were large amounts of *n*-butanol and butyric acid, but 2-butanol and its proposed metabolites were not found. Ethanol was not detected.

There are three possible sources of the compounds identified in these individuals. First, with unusual microbial colonization of the bowel, 3-hydroxy-2-butanone and 2,3-butanediol are produced in the intestine by bacterial fermentation and absorbed (21). In this case, this is an unlikely explanation. Second, 3-hydroxy-2-butanone and 2,3-butanediol (and traces of 2,3-butanedione) have been identified in the blood and urine after ethanol intake (22,23). It is speculated that these derive from condensation of acetaldehyde with pyruvate (23). This could explain the findings in case 1 (Figure 6), but not those in cases 2 and 3. Third, 3-hydroxy-2-butanone and 2,3-butanediol are known metabolites of 2-butanol (24) and 2-butanone (25,26). In this study, 2-butanol and 2-butanone were identified in incubates of *n*-butane with microsomes. It is possible that 2-butanol and 2-butanone may undergo further metabolism to give 2,3-butanediol, 3-hydroxy-2-butanone, and 2,3-butanedione by pathways (Figure 5B), which have been reported for *n*-hexane (5,9,10) (Figure 5A) and *n*-heptane (27). There is little published data for the mammalian metabolism of very short chain hydrocarbons. Further in vitro studies using microsomes, S9 fractions, and liver slices, together with deuterated hydrocarbon substrates, are required.

Table II. Metabolites Identified by HS–SPME–GC–MS after Hydrocarbons Were Incubated (2 h at 37°C) with Activated Rat Liver Microsomes. One Microliter of Liquid (*n*-Hexane and Cyclohexane) or Either 1 or 5 mL of Gas Was Used*

Parent hydrocarbon incubated	Metabolites identified by HS–SPME GC–MS
Propane	2-Propanone (acetone) 2-Propanol
Propene	No metabolites detected
n-Butane	2-Butanone 2-Butanol
Isobutane	No metabolites detected
Butene	3-Butene-2-ol 2-Butene-1ol
cis-2-Butene	3-Butene-2-ol 2-Butene-1ol
trans-2-Butene	3-Butene-2-ol 2-Butene-1ol
n-Hexane	2-Hexanone 1-Hexanol 2-Hexanol 3-Hexanol
Cyclohexane	Cyclohexanone Cyclohexanol

* The chiral configuration of the compounds was not assigned. compounds were extracted and analyzed as described in the experimental section.

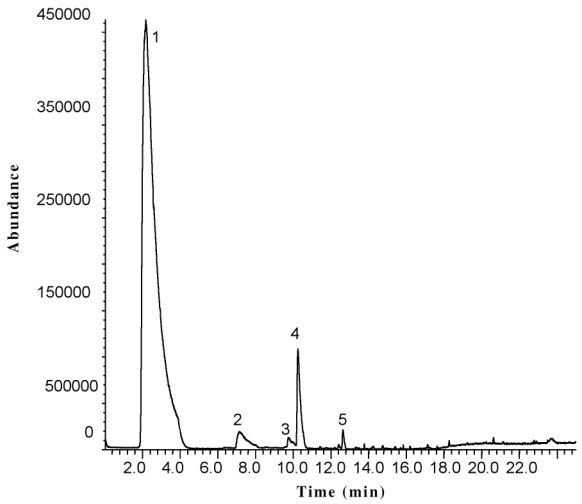

Figure 4. Metabolites detected after incubation of 1 µL of *n*-hexane with microsome/NADPH-generating system (2 h at 37°C). Compounds extracted under the conditions described in the Experimental section. Peak numbers are: *n*-hexane, 1; 2-hexanone, 2; 3-hexanol, 3; 2-hexanol, 4; and 1-hexanol, 5.

SPME has been used to investigate deaths from suspected VSA. Until now, analyses have focused on detection of the parent-abused compounds (3). Because of their volatility and rapid elimination, these substances may not be found in biological specimens (3). Our findings indicate that SPME may be useful for detecting metabolites in autopsy specimens. They also demonstrate the value of the analysis of fat and brain in suspected deaths from VSA. *n*-Butane is known to be sequestered in body fat (20). Our study shows that this is also the case for lipophilic metabolites of hydrocarbons.

These preliminary observations indicate the potential value of SPME for in vitro studies of the metabolism of volatile substances. Advantages over traditional static HS methods are sensitivity and the ability to detect compounds of widely differing polarities simultaneously. In-vial hydrolysis of the conjugates of metabolites can also be achieved simply by adding enzymes (e.g., glucuronidase) or strong acid or alkali as appropriate. Studies using specific enzymes, human microsomal preparations, liver slices, and S9 fractions are feasible. Direct immersion of the SPME fiber into the sample would extend the range of metabolites that can be detected to semivolatile and polar compounds (14). This would also enable investigation of the mammalian metabolism of less volatile substances such as drugs, including barbiturates and antidepressants. However, care is needed to avoid damaging the fiber (14). Current developments (e.g., restricted access materials and molecularly imprinted polymers) in extending the range of fiber chemistries could further increase the value of this approach. The metabolic interaction of compounds could also be studied because abused solvents are generally impure. Toluene, isopropanol, 2-propanone, and 2-butanone have all been suggested to interact with the metabolism of *n*-hexane (5,12,28). Studies with mixtures, however, must allow for different affinities of compounds for the SPME fiber used, as this may influence the metabolic profiles obtained from incubations.

An extension to these studies could be an investigation of the stereochemistry of the metabolism of volatile compounds. One reported study investigated the stereochemistry of the biotransformation of isoprene mono-epoxides and the corresponding diols using rat microsomes (29). In man, isomers produced after anaesthesia with isoflurane have been studied (30). This is an unexplored area for abused hydrocarbons and could lead to a better understanding of the metabolic processes involved.

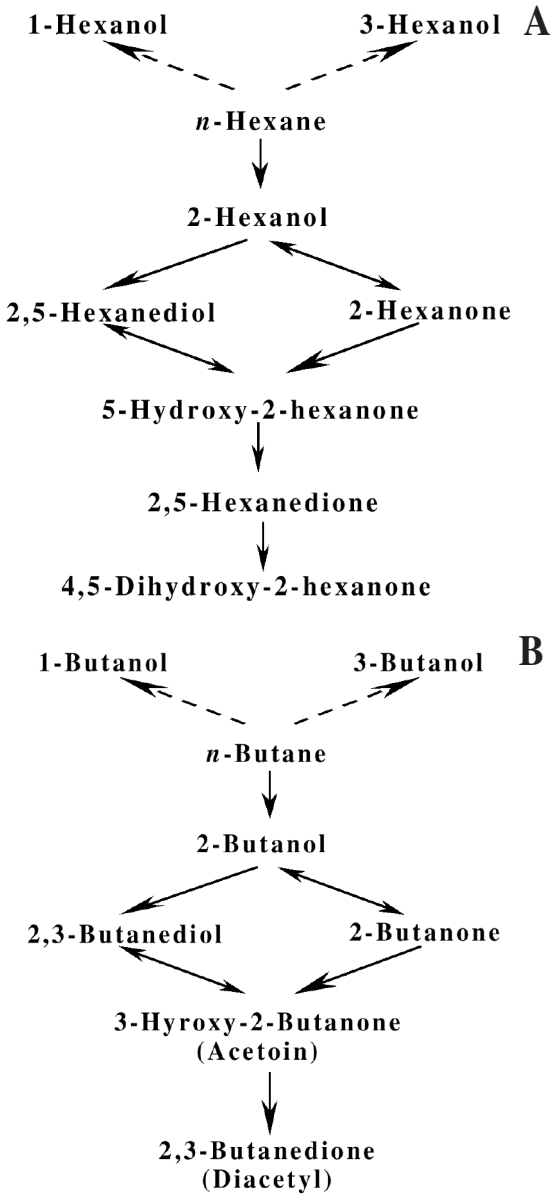

Figure 5. The mammalian metabolism of *n*-hexane (A) and the proposed mammalian metabolism of *n*-butane (B).

Conclusion

This study indicates that HS–SPME–GC–MS is a simple tool to gain further understanding of the metabolism of volatile hydrocarbons that are frequently abused. In addition, the method can

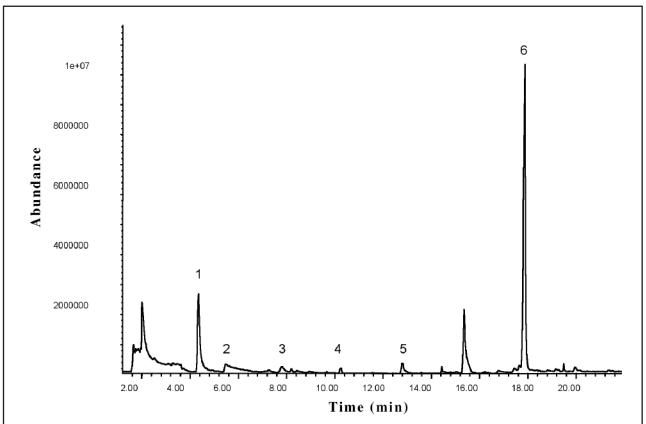

Figure 6. GC–MS profile of HS–SPME extraction of fat from an individual who died suddenly after inhaling cigarette lighter fuel. Fat (2.0 g) was analyzed using a 75-µm thick CAR–PDMS SPME fiber (50°C for 30 min). All conditions are described in the Experimental section, except for the GC oven temperature program: 25°C (held for 5 min), then increased at 5°C/min to 150°C, and then further increased 10°C/min to 220°C (5 min) to improve the separation of the early eluting compounds. Peak numbers are: ethanol, 1; 2,3-butanedione, 2; *n*-propanol, 3; 2-butanol, 4; *n*-butanol, 5; and 3-hydroxy-2-butanone, 6.

be used to confirm exposure in suspected VSA-related deaths. Currently, laboratory diagnosis rests on the detection of the abused parent compound. Identification of specific metabolites of abused substances in biological specimens will extend the window of detection for these short-lived compounds and assist in establishing a cause of death.

One drawback to the use of profiling with HS–SPME is the problem of quantitation of the concentrations of analytes. The HS above a biological sample is usually a complex, multicomponent mixture. Small differences in HS composition can significantly affect the equilibrium, and, in most cases, conventional approaches such as internal or external standardization are not applicable.

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References

1. R.J. Flanagan and T.J. Meredith. "Volatile substances with special reference to volatile substance abuse". In *Human Toxicology*. J. Descotes, Ed. Elsevier, Amsterdam, the Netherlands, 1996, pp. 577–622.
2. R.J. Flanagan. "Volatile substances". In *Clarke's Analysis of Drugs and Poisons*, 3rd ed. A.C. Moffat, M.D. Osselton, and B. Widdop, Eds. Pharmaceutical Press, London, UK, 2004, pp. 227–38.
3. S.M.R. Willie and W.E.E. Lambert. Volatile substance abuse–postmortem diagnosis. *Forensic Sci. Int.* **142:** 135–56 (2004).
4. M.E. Field-Smith, B.K. Butland, J.D. Ramsey, and H.R. Anderson. Trends in deaths associated with abuse of volatile substances 1971–2003. Report 18. London, UK, St. George's Hospital Medical School. http://www.sghms.ac.uk/depts/phs/vsamenu.htm (Date accessed December 15, 2005).
5. World Health Organization. International Programme on Chemical Safety. n-Hexane. *Environ. Health Crit.* **122:** 1–107 (1991). http://www.inchem.org/documents/ehc/ehc/ehc122.htm (Date accessed December 15, 2005).
6. P. Ridgeway, T.E. Nixon, and J.P. Leach. Occupational exposure to organic solvents and long-term nervous system damage detectable by brain imaging, neurophysiology or histopathology. *Food Chem. Toxicol.* **41:** 153–87 (2003).
7. M.J. Prieto, D. Marhuenda, J. Roel, and A. Cardona. Free and total 2,5-hexanedione in biological monitoring of workers exposed to n-hexane in the shoe industry. *Toxicol Lett.* **145:** 249–60 (2003).
8. R.J. Flanagan. "Poisons: inhalants". In *Encyclopaedia of Forensic Sciences*. J. Siegel, Eds. Academic Press, London, UK, 2000, 375–91.
9. R. Toftgård, T. Haaparanta, L. Eng, and J. Halpert. Rat lung and liver microsomal cytochrome P-450 isoenzymes involved in the hydroxylation of n-hexane. *Biochem. Pharmacol.* **35:** 3733–38 (1986).
10. S.J. Crosbie, P.G. Blain, and F.M. Williams. Metabolism of n-hexane by rat liver and extrahepatic tissues and the effect of cytochrome P-450 inducers. *Human Exp. Toxicol.* **16:** 131–37 (1997).
11. B. Mortensen, T. Løkken, K. Zahlsen, and O.G. Nilsen. Comparison and in vivo relevance of two different in vitro head space metabolic systems: liver S9 and liver slices. *Pharmacol. Toxicol.* **81:** 35–41 (1997).
12. B. Mortensen, K. Zahlsen, and O.G. Nilsen. Metabolic interaction of n-hexane and methyl ethyl ketone in vitro in a headspace rat liver S9 vial equilibrium system. *Pharmacol. Toxicol.* **82:** 67–73 (1998).
13. J. Pawliszyn. *Solid-Phase Microextraction: Theory and Practice.* Wiley-VCH, Chichester, UK, 1997.
14. G.A. Mills and V. Walker. Headspace solid-phase microextraction procedures for gas chromatographic analysis of biological fluids and materials–a review. *J. Chromatogr. A* **902:** 267–87 (2000).
15. G.A. Mills and V. Walker. Headspace solid-phase microextraction profiling of volatile compounds in urine: application to metabolic investigations. *J. Chromatogr. B* **753:** 259–68 (2001).
16. B. Mortensen and O.G. Nilsen. Optimization and application of the head space liver S9 equilibration technique for metabolic studies of organic solvents. *Phamacol. Toxicol.* **82:** 142–46 (1998).
17. J.M. Olson, S.G. Kim, C.A. Reidy, J.T. Johnson, and F.N. Novak. Oxidation of 1,1,1,2-tetrafluroethane in rat liver microsomes is catalyzed primarily by cytochrome P450IIEI. *Drug Metab. Dispos.* **19:** 298–303 (1990).
18. T.H. Elliott, D.V. Parke, and R.T. Williams. Studies in detoxication 79. The metabolism of cyclo[14C] hexane and its derivatives. *Biochem. J.* **72:** 194–200 (1959).
19. C.S. Lieber. The discovery of the microsomal ethanol oxidizing system and its physiologic and pathologic role. *Drug Metab. Rev.* **36:** 511–29 (2004).
20. European Agency for the Evaluation of Medicinal Products, Committee for Veterinary Medicinal Products. "Propane, *n*-butane, and isobutane". Summary report. EMEA/MRL/031/95. http://emea.eu.int/pdfs/vet/mrls/00319.pdf (accessed December 18, 2005).
21. G.A Mills, and V. Walker. Urinary excretion of 2,3-butanediol and acetoin by babies on a special care unit. *Clin. Chim. Acta* **179:** 51–60 (1989).
22. J.A. Montgomery, M. Jetté, and H. Brunengraber. Assay of physiological levels of 2,3-butanediol diastereomers in blood and urine by gas chromatography-mass spectrometry. *Anal. Biochem.* **185:** 71–76 (1990).
23. M. Otsuka, N. Harada, T. Itabashi, and S. Ohmori. Blood and urinary levels of ethanol, acetaldehyde, and C_4 compounds such as diacetyl, acetoin, and 2,3-butanediol in normal male students after ethanol ingestion. *Alcohol* **17:** 119–24 (1999).
24. F.K. Dietz, M. Rodriguez-Giaxola, G.J. Traiger, V.S. Stella, and K.S. Himmelstein. Pharmacokinetics of 2-butanol and its metabolites in the rat. *J. Pharmokinet. Biopharm.* **9:** 553–76 (1981).
25. S. Kezic and A.C. Munster. Determination of methyl ethyl ketone and its metabolites in urine using capillary gas chromatography. *J. Chromatogr.* **428:** 275–80 (1988).
26. J. Liira, V. Riihimaki, and P. Pfaffli. Kinetics of methyl ethyl ketone in man: absorption, distribution and elimination in inhalation exposure. *Int. Arch. Occup. Environ. Health* **60:** 195–200 (1988).
27. L. Perbellini, F. Brugnone, V. Cocheo, E. De Rosa, and G.B. Bartolucci. Identification of the n-heptane metabolites in rat and human urine. *Arch. Toxicol.* **58:** 229–34 (1986).
28. X. Yu, G. Johanson, G. Ichihara, E. Shibata, M. Kamijima, Y. Ono, and Y. Takeuchi. Physiologically based pharmacokinetic modeling of metabolic interactions between n-hexane and toluene in humans. *J. Occup. Health.* **40:** 293–301 (1998).
29. C. Chiappe, A. De Rubertis, V. Tinagli, G. Amato, and P.G. Gervasi. Stereochemical course of the biotransformation of isoprene monoepoxides and of the corresponding diols with liver microsomes from control and induced rats. *Chem. Res. Toxicol.* **13:** 831–38 (2000).
30. H.A. Haeberle, H.G. Wahl, H. Jakubetz, H Krause, R. Schmidt, V. Schurig, and H.-J. Dieterich. Accumulation of S(+) enantiomer in human beings after general anaesthesia with isoflurane racemate. *Eur. J. Anaesthesiol.* **19:** 641–46 (2002).

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