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TECHNICAL NOTE

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Solvent-free sample preparation by headspace solid-phase microextraction applied to the tracing of n-butyl nitrite abuse

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Abstract The most common alkyl nitrites encountered in forensic toxicology are iso-butyl, n-butyl and iso-pentyl(amyl) nitrites. All have become popular as an aphrodisiac, especially among the homosexual population. Alkyl nitrites are a volatile and unstable group of compounds, which hydrolyse in aqueous matrices to the alcohol and nitrite ion. Here we describe a fast, clean and sensitive procedure for the detection of hydrolysed n-butyl nitrite in whole human blood using a new, solvent-free sampling technique, the headspace solid-phase microextraction (HSPME), combined with GC/FID analysis. Sample preparation was investigated using two different stationary phases (100 µm polydimethylsiloxane and 85 µm polyacrylate), coating a fused silica fibre. The effect of different sampling times at fixed temperatures was also studied. Our results demonstrate that the HSPME/GC/FID procedure allows tracing of n-butyl nitrite abuse and detects hydrolysed n-butyl nitrite, i.e. released n-butanol, in whole blood at the 1 ng/mL level.

Key words Headspace · Solid-phase · Microextraction · N-butyl nitrite · Chromatography

Introduction

In the analysis of aqueous samples, e.g. human blood and urine, sample preparation is frequently the most time-consuming step and represents the primary cause of analyte loss from the matrix. Nowadays, sampling of volatile organic compounds (VOCs) is often achieved using either headspace or purge-and-trap methods (e.g. [1]). Disadvantages of the first method are, inter alia, the confinement to concentrated samples, and those of the latter method are the frequent occurrence of leaks, contaminated traps, and cost. Ideally, the sample preparation technique for VOC should be solvent-free, simple, inexpensive, efficient, selective and compatible with a wide range of separation methods and applications (for a review see [2]).

The headspace solid-phase microextraction technique (HSPME), developed by Zhang and Pawliszyn [3] meets most of the aforementioned criteria. HSPME is a solvent-free sample preparation technique in which a fused silica fibre, coated with a polymeric organic stationary phase, is introduced into the headspace above the sample (Fig. 1). Then, VOCs are extracted and concentrated on the coating for the purpose of consecutive desorption, separation and analysis in an analytical instrument, e.g. gas chro-

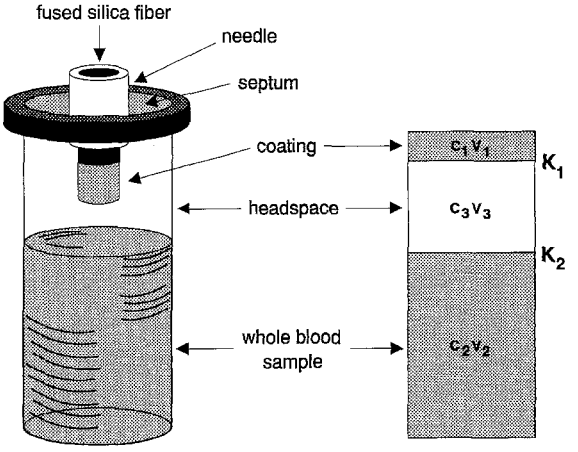

Fig. 1 The headspace solid-phase microextraction (HSPME) technique. A diagram of the HSPME technique is shown on the left, together with a one-dimensional representation of the three-phase system on the right. K_1 and K_2 are the coating/headspace and headspace/blood partition coefficients, respectively; C_1, C_2, and C_3 are the concentrations of the analyte in the coating, whole blood, and headspace, respectively; V_1, V_2, and V_3 are the volumes of the coating, whole blood, and headspace, respectively

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matograph (GC). HSPME represents a modification of direct SPME, where the coated fused silica fibre is exposed directly to the aqueous sample (e.g. [4]). In both HSPME and SPME sampling techniques, analytes establish an equilibrium between the sample matrix and a stationary phase coated on the fused silica fibre. However, the main advantage of HSPME is the possibility to extract VOCs from very complex matrices such as sludge, clay soil, and also whole (human) blood. Because no solvent is involved, and the analytes are rapidly desorbed onto the capillary GC column, minimum detection limits can be improved and high resolution can be maintained.

The objective of this study was to develop a fast, clean and sensitive procedure for the tracing of alkyl nitrites abuse, using the HSPME technique combined with capillary GC/FID analysis. Alkyl nitrite inhalants are frequently abused substances, primarily by homosexual men and people who use nitrites to facilitate sexual intercourse and/or produce euphoria (for review and several cases of abuse, see [5, 6]). Most alkyl nitrites are colourless or yellow liquids at room temperature and are highly volatile. They are esters of nitrous acid that have a fruity odour and have been nicknamed 'poppers', because of the sound made when glass capsules containing amyl nitrite are crushed [7]. These VOCs are an unstable group of compounds, which hydrolyse in aqueous matrices to the alcohol and nitrite ion. In whole blood, the released nitrite is also quickly converted to nitrate with the appearance of methemoglobin [8]. Scientific interest in nitrites increased in the 1980s due to their possible links to AIDS. Currently, at least four hypotheses have been proposed which suggest a role for nitrites in the pathogenesis of AIDS [5, 6]. Therefore, discouraging the use of nitrite inhalants continues to be a worthwhile public health goal. In this scope, we report here a fast, clean and sensitive HSPME procedure for the tracing of n-butyl nitrite abuse. n-Butyl nitrite was chosen, because it is not subject to specific regulation by the FDA (in contrast to prescription requirement for amyl nitrite), it is readily available as incense or room odorizer, and butyl nitrites are presently the most widely abused sexual stimulants among the alkyl nitrites.

Methods

Headspace solid-phase microextraction (HSPME)

Samples containing 5 ng/5 mL–1 mg/5 mL of n-butyl nitrite were prepared from a fresh stock containing 5 mg n-butyl nitrite in 5 mL human blood in a 10 mL vial (Fig. 1). The vial was sealed with a teflon-faced black rubber septum and plastic cap. The internal standard (IS) was n-propanol in a concentration of 5 ng/5 mL or 1 mg/5 mL. Control runs with both n-butanol and n-propanol were performed to check the relative retention time of n-butanol originating from the hydrolysed n-butyl nitrite in the other samples. All samples were equilibrated for 1–3 h at room temperature (20–23° C), prior to HSPME during 5–20 min at 60° C. The adjustable needle guide/depth gauge of the HSPME fibre holder (Supelco, Bellefonte, PA) was positioned at 1.8 cm. The analytes, i.e. hydrolysed butyl nitrite and n-propanol (IS), were absorbed directly from whole blood under unstirred conditions by headspace extraction onto a phase-coated fused silica fibre. Two different stationary phases were used and compared: 100 µm polydimethylsiloxane and 85 µm polyacrylate (Supelco, Bellefonte, PA). Conditioning of the fibres was performed at 155° C for at least 30 min prior to sampling of the analytes. To obtain absorption-time profiles, the effect of different sampling times at 60° C was also investigated. All tested fibres were reusable, provided that they were given good care.

Gas chromatography-flame ionisation detector (GC/FID)

The fibre was inserted manually into the injection port of the GC (HP 5890 Series II, Avondale, PA) for thermal desorption during 1 min in the splitless mode. The HSPME holder was positioned at 2 cm. The temperature of the injector, oven, and detector was set at 240, 50, and 260° C, respectively. The oven was programmed to hold the initial temperature of 50° C for 5 min, followed by a ramp of 10° C/min to 260° C, and held for 5 or 10 min. The separation was performed on a capillary HP-1 fused silica column (30 m × 0.53 mm) coated with a 2.65 µm methylsilicone film, and He carrier gas with a velocity of 4 mL/min at 100° C (0.2 kg/cm^2 pressure). The inception of the column was cooled with liquid CO_2 gas to a temperature between –5 and 5° C in order to condense and concentrate the analytes [2]. FID was operated with helium make-up of 15 mL/min, hydrogen 12 mL/min, and air 250 mL/min. Signal output was attenuated with range-values of 1 and attenuation-values of 1–10. Registration of the data was performed with a pen recorder (10 mm/min paper speed, 1 mV full scale; Kipp, A41, Delft, Holland).

Results

HSPME consists of two processes: i) partitioning of the analytes between the polymeric coating and the sample, and ii) desorption of the concentrated analytes into an analytical instrument (for reviews see [2, 3]). The amount of analytes absorbed by the stationary phase, i.e. the polymeric coating on the fibre, is related to the overall equilibrium of analytes in the three-phase system: human blood, headspace and coating (Fig. 1). Since the total amount of an analyte should be the same during the extraction, we have:

$$C_0V_2 = C_1^{\infty}V_1 + C_2^{\infty}V_2 + C_3^{\infty}V_3$$

where C_0 is the initial concentration of the analyte in the human blood; C_1^{∞}, C_2^{∞}, and C_3^{∞} are the equilibrium concentrations of the analyte in the coating, the whole blood, and the headspace, respectively; V_1, V_2, and V_3 are the volumes of the coating, the whole blood, and the headspace, respectively. With the coating/headspace partition coefficient equal to $K_1 = C_1^{\infty}/C_3^{\infty}$, and the headspace/blood partition coefficient equal to $K_2 = C_3^{\infty}/C_2^{\infty}$, the amount of the analyte absorbed by the coating can be expressed as:

$$n = \frac{C_0V_1V_2K_1K_2}{K_1K_2V_1 + K_2V_3 + V_2}$$

To characterise the performance of the fibres, several parameters were studied: sensitivity, reproducibility, absorption-time profile and comparison of two types of fibre coating. A representative chromatogram obtained with the HSPME technique using a 85 µm polyacrylate fibre is shown in Fig. 2. Panel A illustrates the control chromatogram obtained from 5 mL whole human blood (i.e.

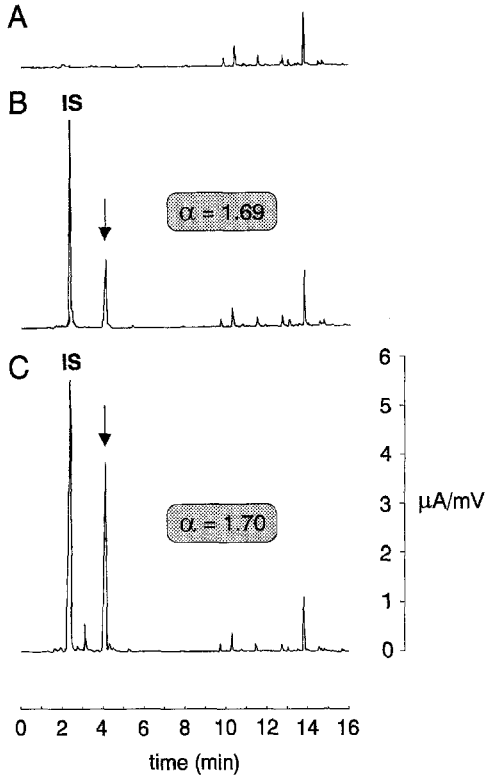

Fig. 2A–C Separation and detection of 200 ppm n-propanol and n-butanol. **A** Control chromatogram obtained from 5 mL whole human blood under negative conditions with a 85 µm polyacrylate fibre coating. **B** Separation and detection of 200 ppm n-propanol, as internal standard (IS), and n-butanol (*arrow*). The relative retention time, α, for n-butanol was 1.69. **C** Separation and detection of 200 ppm n-propanol (IS) and n-butanol (*arrow*), originating from the hydrolysed n-butyl nitrite added to the 5 mL blood sample. The same relative retention time, α = 1.70, was obtained

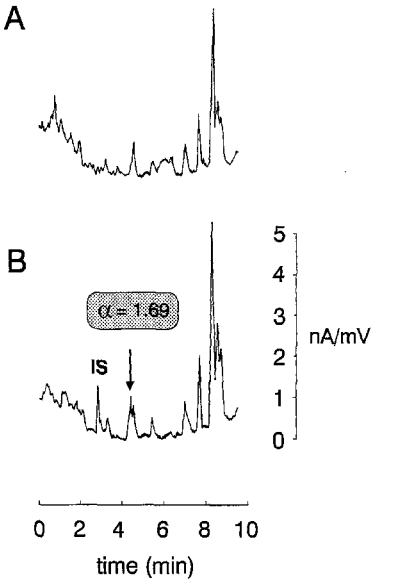

Fig. 3A, B Separation and detection of 1 ppb n-propanol and n-butanol. **A** Control chromatogram obtained from 5 mL whole human blood under negative conditions with a 100 µm polydimethylsiloxane fibre coating. **B** Separation and detection of 1 ppb n-propanol (IS) and n-butanol (*arrow*). The same relative retention time, α = 1.69, was obtained as in Fig. 2

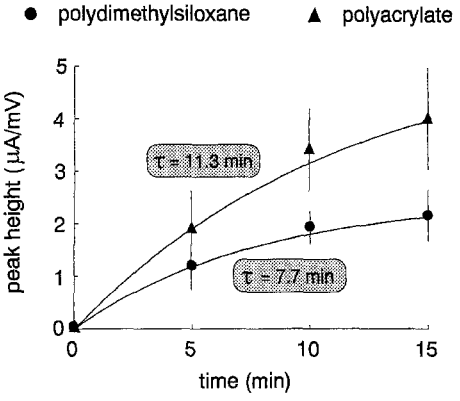

Fig. 4 Absorption-time profiles. Absorption-time profiles were constructed for the 100 µm polydimethylsiloxane (*filled circles*) and 85 µm polyacrylate (*filled triangles*) coated fibres by monitoring peak heights of n-butanol as a function of sampling time at 60° C. The results are pooled data from at least 3 experiments for each sampling time and are shown as means ± S.E.M. The time constants were obtained after fitting an exponential to the data (see Results). The maximum peak height obtained with the polyacrylate phase was significantly higher than the one obtained with the polydimethylsiloxane phase

negative control). Addition of 200 ppm of the internal standard (IS; n-propanol) and n-butanol to whole blood, with absorption/desorption to/from the fibre and consecutive column separation led to the appearance of two clear peaks, corresponding to the analytes (panel B). The relative retention time, α, of the second peak was 1.69. Next, a sample containing 200 ppm n-butyl nitrite plus n-propanol (IS) was subjected to the same procedure (panel C). Two separate peaks with the same retention times as in panel B could be detected in the chromatogram ($\alpha_{\text{second peak}}$ = 1.70). This points to the presence of n-butanol, which originates from the hydrolysed n-butyl nitrite. By repeating this experiment several times (n > 5), we could ascertain the experimental reproducibility.

The sensitivity of the HSPME/GC/FID procedure described here is characterized by a detection level of hydrolysed alkyl nitrites in whole blood at the 1 ng/mL level (1 ppb). A representative chromatogram is shown in Fig. 3. Panel A illustrates the control chromatogram from 5 mL human blood under control conditions after headspace sampling with a 100 µm polydimethylsiloxane fibre. Panel B shows the detection of 5 ng n-propanol (IS), together with a small peak characterized by a relative retention time of 1.69. This peak points to the presence of n-butanol, originating from the 5 ng n-butyl nitrite in the 5 mL human blood sample.

Next, we constructed absorption-time profiles by monitoring peak heights of n-butanol as a function of sampling time at 60° C. In addition, the effect of two different stationary phases, polydimethylsiloxane versus polyacrylate, was studied. Figure 4 illustrates pooled data from at least three experiments for each sampling time given in the X-axis. It can be seen that equilibrium of analyte extraction with 100 µm polydimethylsiloxane coating was

more rapidly established than with 85 μm polyacrylate coating. Time constants characterising the time dependence of absorption were obtained after fitting an exponential to the data with equation:

$$\text{peak height} = A * (1-\exp(-t/\tau)) + C$$

with A the maximal peak height, t the absorption time, τ the time constant, and C a constant. Values for τ were 7.7 min and 11.3 min for polydimethylsiloxane and polyacrylate, respectively. The maximal peak height obtained with the polyacrylate coating (A = 5.44 μA/mV) was significantly higher than the one obtained with polydimethylsiloxane coating (A = 2.52 μA/mV). This indicates that the polyacrylate phase more successfully extracts polar analytes, such as low molecular weight alcohols.

We also investigated whether pre-incubation at 37° C of the vials had any influence on the detection of hydrolysed nitrites. We have not observed any significant difference between vials placed for 1 h at room temperature versus 1 h at 37° C, preceding the incubation for 15 min at 60° C. In contrast, conditioning of the fibres at temperatures in the range of 150–240° C improved the HSPME sampling efficiency of the fibre coating for the analytes, as opposed to no conditioning (i.e. room temperature). However, we have not seen any significant difference in chromatograms obtained with conditioned fibres at temperatures 155° C or 240° C.

Discussion

For VOCs, it is known that the release of analytes into the headspace is relatively easy because analytes tend to vaporise once they are dissociated from their matrix [1–3]. For semi-volatile compounds, the low volatility and relatively large molecular size may slow the mass transfer from the matrix to the headspace, resulting in a longer extraction time. In HSPME, the mass transfer from the (aqueous) matrix to the headspace can be speeded up by constantly stirring the sample to generate a continuously fresh surface. In addition, kinetic limitations of the extraction procedure can also be overcome by heating the sample, which increases the vapour pressure of analytes and provides the energy necessary for the analytes to be dissociated from the matrix. However, as the sampling temperature increases, the fibre coating begins to lose its ability to absorb analytes. Thus, there is an optimum temperature for HSPME. It has also been shown for direct SPME that, by adding a salt to the aqueous sample (NaCl or Na_2SO_4 to increase the ionic strength of the solution), the partitioning of polar organic compounds into the polymer coating of the SPME fibre can be increased [2]. In our protocol, the headspace sampling procedure was always carried out at a temperature of 60° C (for 5–20 min sampling time), but without stirring the blood samples and without adding a salt.

One of the major features of the HSPME is that the volume of the fibre coating (V_1, see Fig. 1) is extremely small, approximately 10^{-4} cm^3 [3]. For VOCs, the amount of analyte extracted by the coating is insignificant compared to the amount of analyte existing in the headspace. Thus, the concentration in the aqueous solution, i.e. whole human blood, is virtually unchanged during the extraction. As a result, the extraction time is determined largely by diffusion in the headspace phase. Furthermore, the selective absorption of the fibre coating prevents a substantial amount of oxygen and moisture from getting into the GC column, representing a major advantage over previous headspace techniques.

Thermal desorption of analytes from the SPME coating is also very effective: as temperature increases, the coating/gas partition coefficients decrease and the ability of the coating to retain analytes diminishes quickly (240° C injector in our case). The constant flow of carrier gas within the GC also facilitates the removal of analytes from the coating. These features eliminate carry-over of material on the fibre coating between consecutive analyses. This is of paramount importance when analysing two samples successively, with the first sample being highly concentrated with analytes in contrast to the second one. We also found that within-run and between-run precision was very high, which can be explained by the effective absorption and desorption of the analytes according to the experimental conditions. In order to generate a very narrow band of analytes in the column, the inception of our HP-1 column was cooled with liquid CO_2 gas (–5 and 5° C), which condensed the analytes [2].

Absorption-time profiles are often characterized by an initial rapid rise, associated with partitioning of analytes originally present in the gaseous headspace, followed by a section with a smaller slope, determined by slower mass transport of analytes from the matrix [3]. Our absorption-time profiles could adequately be described by a single exponential, from which it can be interpreted that the original concentration of analytes in the headspace was minimal. The fact that the maximal peak height obtained with the polyacrylate coating was significantly higher than the one obtained with the polydimethylsiloxane coating, indicates that the polyacrylate coating more successfully extracts polar analytes, such as alcohols of low molecular weight. These findings corroborate the documentation provided by the manufacturer on the SPME fibres and their coating material (Supelco, Bellefonte, PA). Very recently, a third type of coating has also been commercialised by this company (a 65 μm carbowax/divinyl-benzene coating) for extraction of alcohols from samples at ppm to ppb concentrations. The polyacrylate-coated fibre we have used also especially overcomes the problems of extracting polar analytes from a polar matrix (e.g. extraction of phenols from water). For nonpolar and moderately polar organic compounds, it is known that conventional coatings are capable of extracting at least a few picograms of analyte from low part-per-trillion samples, a level that typically constitutes the detection limits specified by regulatory agencies (for reviews see [2, 3]). Using SPME/GC/FID analysis for benzene, toluene, ethylbenzene, and xylene isomers, similar limits of detection (0.3 μg/L) as in our study have been reported [9]. The deter-

mination of the detection limit of 1 ppb may appear to be variable dependent on the underlying background peak seen in Fig. 3. Therefore we repeated this experiment very carefully ($n > 5$). As we could never detect a peak with a relative retention time of 1.69 in our negative blood samples, in contrast to all the samples containing at least 1 ppb or more hydrolysed n-butylnitrite, we are confident of a detection limit of 1 ppb.

There are few limitations of the HSPME technique. One current limitation, for instance, is the confinement to the extraction of volatile and semi-volatile compounds with boiling temperatures substantially above those of the solvents. However, continued development of new sorbents, e.g. in combination with GC/MS, will be very important for sample preparation and detection of a wide range of organic and inorganic compounds in the near future [2]. Another point which should be kept in mind is the possibility that other volatiles might be present in whole blood due to improper storage, postmortem alcohol production, or consumption of alcoholic beverages (e.g. 1-propanol, 2-propanol, 1-butanol, 2-butanol, iso-butanol, tert-butanol, acetaldehyde, and acetone) [10, 11]. In order to avoid potential problems, adequate storage at a maximum temperature of 4°C in the presence of 1–2% w/v sodium fluoride is a prerequisite [12, 13]. In case of de novo alcohol production, the chromatogram will be characterized by several peaks, corresponding to the different volatiles present in the sample (e.g. alcohols and aldehydes). In case of consumption of alcoholic beverages, a predominant ethanol peak will always be discernible, eclipsing the other peaks. Therefore, the abovementioned cases will produce typical chromatograms, different from those where only hydrolysed n-butyl nitrite, i.e. n-butanol, is detected. Furthermore, the outcome of a HSPME/GC/FID analysis, pointing to the absence or presence of alkyl nitrites in whole blood, will usually fit in the scope of clear and easily recognizable symptoms of the intoxicated person, which can be linked to volatile nitrite abuse [5, 6].

In conclusion, this technical note describes how the HSPME technique can integrate sampling, extraction, concentration, and sample introduction of hydrolysed n-butyl nitrite in whole human blood. The salient feature of our sample preparation protocol is the solvent-free extraction, which is simple, inexpensive, fast, clean, and sensitive. For forensic analyses, we believe that HSPME offers a key method for absolute confirmation of biological specimens.

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