Rapid Method for Determination of Residual *tert*-Butanol in Liposomes Using Solid-Phase Microextraction and Gas Chromatography

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Abstract

A simple, rapid, and reliable method to detect residual levels of tert-butanol in liposomes using sec-butanol as an internal standard has been developed. Solid-phase microextraction (SPME) followed by gas chromatographic analysis was used to quantify the amount of residual tert-butanol in freeze-dried liposome material. Only 1 min was necessary for reproducible amounts of analyte to absorb onto the SPME fiber, and because this method requires very little sample preparation, a single analysis can be completed in less than 15 min. This method had a linear range of 10–600 µg/mL. Careful control of times of temperature equilibration and exposure to headspace was necessary to ensure reproducible results. This method can easily be applied to other applications in the food and pharmaceutical industries where detection of residual solvents, such as hexane and chloroform, is necessary.

Introduction

In recent years, the field of liposome technology has grown rapidly, primarily because of the usefulness of liposomes as drug carriers (1). Liposomes are ideal carriers for many drugs because they allow site-specific treatment, increasing the therapeutic effectiveness of the drug. They can also direct treatment away from areas that may be more sensitive to toxic effects of drugs. In order for these drugs to be marketed, they must succeed in clinical trials, where success hinges on both the chemical and physical stability of the drug and carrier. Aqueous liposome solutions are prone to stability problems, including aggregation, phospholipid hydrolysis, and leakage of the drug being carried, all of which can lead to a decreased shelf life (2,3). To avoid these stability problems, freeze-drying of liposomes has become common practice. It has been shown that the addition of organic solvents can increase the rate of ice sublimation, thus decreasing the length of the freeze-drying process (3). *tert*-Butanol is a particularly useful solvent for these applications because its high melting point of 25°C allows freeze-drying to be conducted at room temperature. Lipids are also four to five times more soluble in *tert*-butanol than in other organic solvents (4). Thus, the use of *tert*-butanol in liposome preparation is gaining popularity (2,4–6).

Under the ICH Guidelines for Residual Solvents (7), *tert*-butanol is classified as a solvent with low toxic potential and of low risk to human health. In accordance with this, the current US Pharmacopeia (8) regulations have classified *tert*-butanol as a class 3 chemical of low toxicity. The new monograph permits the consumption of *tert*-butanol up to levels of 50 mg per day, corresponding to a concentration of 5000 µg/mL or 0.5% in a liposome preparation. Regulatory requirements dictate that levels > 0.5% be accurately quantified, often with product-specific techniques requiring considerable method development. Though it seems logical that the use of a small amount of *tert*-butanol during freeze drying would result in lower levels of residual *tert*-butanol, Wittaya-Areekul and Nail (9) showed that a higher initial *tert*-butanol level promotes crystallization of the *tert*-butanol hydrate/ice eutectic mixture, resulting in lower residual *tert*-butanol levels. Because it is very difficult to remove all traces of *tert*-butanol from the product, it becomes important to quantify the residual solvent remaining to ensure compliance with regulatory requirements.

Gogineni et al. (4) developed a gas chromatography (GC) method to detect residual levels of *tert*-butanol in freeze-dried liposomes; however, the sample preparation required for that method can be time consuming. As an alternative, we examined methods to quantify the amount of *tert*-butanol remaining in liposome mixtures after freeze drying using solid-phase microextraction (SPME) to concentrate the analyte and GC with flame

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ionization detection (FID) for quantification. Although both methods allow for rapid GC analysis, the important difference between the two is the time required for sample preparation. The method discussed here requires very little sample preparation, making it quicker and easier to perform. By minimizing sample handling, SPME also reduces the risk of analyte loss and inaccurate results. Several studies have described the use of SPME-GC to identify and quantify other residual chemicals (e.g., 10–14) and Camaruso et al. (11) identified GC as the most popular method for analyzing for residual solvents in the pharmaceutical industry. SPME–GC has been used since the 1990s to detect volatile components in food products including cheese, wine, and seafood (15,16,17). It has also been used to analyze volatile compounds in air and water samples (18,19). The common approach with SPME is to optimize the procedure for maximal recovery of the analyte of interest, with reproducible recoveries typically in the range of 10 to 100 ng/mL or ~ 10,000 to 100,000 times lower than necessary for regulatory compliance. Such low detection limits are usually achieved at the expense of total analysis time. In a recent review, Camarasu et al. (11) noted the growing need to monitor residual solvent with on-line methods during processing. This requires rapid methods that measure volatiles at levels relevant for regulatory compliance. Here, we present such an SPME-GC method to determine residual levels of *tert*-butanol in freeze-dried liposomes, where speed of analysis, rather than recovery, is the main goal.

Experimental

Materials

sec- and *tert*-Butanol (99% pure) were obtained from Sigma Chemical Co. (Oakville, ON, Canada). Phosphate-buffered saline (pH 7.4) was obtained from Invitrogen (Carlsbad, CA.). SPME fibers (100 µm polymethylsiloxane), an SPME fiber holder for manual sampling, 22-mL glass vials, polytetrafluoroethylene–silicone rubber septa, and phenolic screw caps were purchased from Supelco (Oakville, ON, Canada). A custom-made heating block designed to accommodate 22-mL glass vials was used to control temperature. A proprietary liposome mixture composed of only phosphatidyl choline and cholesterol in phosphate buffered saline was donated. Solvent was not used in the preparation of this sample, so it was known to be free of *tert*-butanol. A test sample of the same liposome material prepared using freeze-drying with *tert*-butanol was also donated.

Standard Curve

Exactly 1.0 mL of liposome mixture, and 1.0 mL of *sec*-butanol (1.03 mg/mL in phosphate buffered saline) as an internal standard were added to a 22-mL glass vial containing a 1-cm stir bar. Varying amounts of *tert*-butanol in phosphate buffer with volumes ranging from 0.500–10.00 mL were added to create a set of standards. The mixture was then diluted to exactly 16.0 mL (see Table I for final concentrations). The *tert*-butanol concentrations were calculated to produce a curve with an upper limit corresponding to 625 µg/mL in 16.0 mL or 10000 µg in a 1 mL liposome sample (i.e., 10,000 µg/16 mL = 625 µg/mL; Table I). This generated an upper limit of the standard curve that was twice the allowable *tert*-butanol level of 5000 µg/mL in a 1-mL liposome sample. Vials were then capped with phenolic screw caps containing PTFE/silicone rubber septa. Samples were stirred at 200 rpm and heated at 30°C for 5.0 min. After the 5 min equilibration time, the SPME fiber was manually inserted into the vial at a depth of 1.4 cm and exposed to the sample headspace for 1.0 min. This 5-min period was the minimum time required for the liposome mixture to reach 30°C, assuming an ambient temperature no lower than 20°C. Each concentration was tested in triplicate.

Instrumentation

Extracted volatile compounds were analyzed using a Varian (Palo Alto, CA) 3400 GC equipped with an FID. This was accomplished by manually inserting the SPME fiber into the injector port (splitless mode, 250°C; 0.75 mm liner) to a depth of 3.6 cm. The fiber was left in the injector for the duration of the GC run. Before each use, a blank SPME fiber was run to desorb any volatiles that may have accumulated between samples. Volatile analytes desorbed from the fiber were separated on a SAC-5 column (30 m × 0.25 mm × 0.25 µm film thickness, Supelco, Oakville, ON, Canada). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The oven temperature was held at 40°C for 5 min and was then increased at a rate of 30°C /min to 190°C

Table I. Calibration Data Used to Create Standard Curve; Both Individual Data Points and the Means (± sd) Are Shown*

Calibration Point	*tert*-butanol in liposome (µg/mL)	*tert*-butanol in 16.0 mL buffer		*sec*-butanol in 16.0 mL buffer		*tert*-butanol area / sec-butanol area
		(µg/mL)	area (counts)	(µg/mL)	area (counts)	
1	160	10.0	2806	64.4	29457	0.10
1	160	10.0	4107	64.4	30168	0.14
1	160	10.0	3146	64.4	29522	0.11
			3353 ± 674		29715 ± 393	0.11 ± 0.02
2	499	31.2	9114	64.4	30446	0.30
2	499	31.2	9398	64.4	31629	0.30
2	499	31.2	11937	64.4	33041	0.36
			10149 ± 1554		31705 ± 1299	0.32 ± 0.04
3	4990	312	97801	64.4	32699	2.99
3	4990	312	95065	64.4	31282	3.04
3	4990	312	106584	64.4	34435	3.10
			99816 ± 6018		32805 ± 1579	3.04 ± 0.05
4	10000	625	185120	64.4	31146	5.99
4	10000	625	188180	64.4	32028	5.88
4	10000	625	175207	64.4	30930	5.63
			182835 ± 6781		31368 ± 581	5.83 ± 0.18

* Sample conditions were held constant throughout standard curve development. *Tert*-butanol concentrations in liposome are based on analysis of a 1.0 mL sample. This data generated a standard curve with equation: (area ratio) = 0.00058(*tert*-butanol in liposome) + 0.047 (r^2 = 0.998).

with a total run time of 10 min. The FID was maintained at 300°C. *tert*- and *sec*-Butanol were identified by comparison of retention times to known standards.

Results and Discussion

When regression was performed on the data generated from the standard curve (Table I), the residual sums of squares was found to be 0.998 and the standard error was 0.104. The high residual sum of squares, combined with the low standard error, demonstrates adequate fit for quantitative determination. Considerable variation in repetitive analysis of the same sample is obvious (Table I); however when the *tert*-butanol peak areas vary, the *sec*-butanol area follows suit. Because we are only interested in the ratio of the peak area of *tert*-butanol to *sec*-butanol, a change in area counts of any individual peak is not problematic but, rather, expected. This clearly illustrates the necessity of using internal standards when employing SPME in a quantitative manner. *sec*-Butanol is a good choice of internal standard for this application because it has a similar vapor pressure and polarity as *tert*-butanol, providing comparable absorption to the SPME fiber.

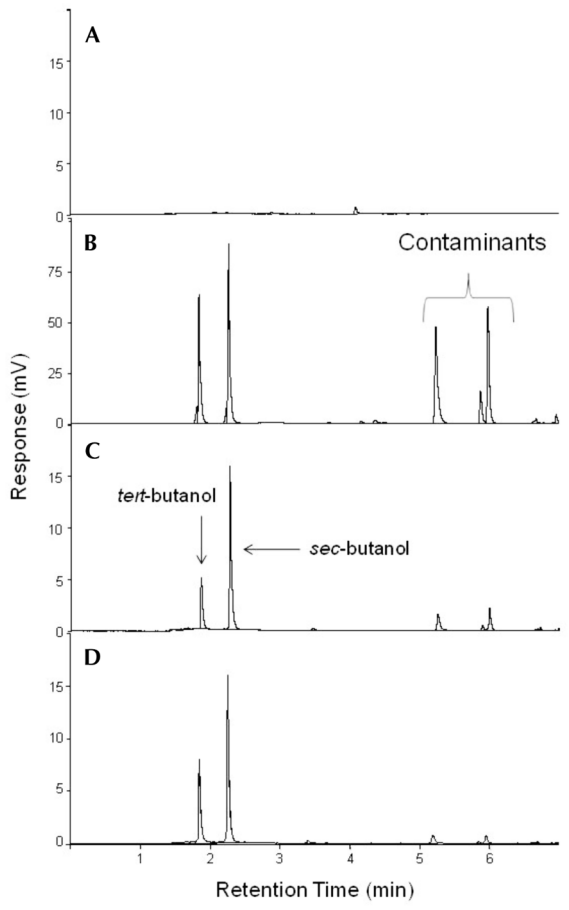

Figure 1. Chromatograms of (A) phosphate buffer only; (B) *tert*-butanol (312 µg/mL) and *sec*-butanol standard (64.4 µg/mL) with a 20.0 min exposure time; (C) *tert*-butanol (312 µg/mL) and *sec*-butanol standard (64.4 µg/mL) with a 1.0 min exposure time. Note the increase in response scale; and (D) test sample of a commercial liposome preparation.

As originally noted by Gogineni et al. (4), the challenge in quantitatively determining *tert*-butanol with GC stems from its low boiling point. Normally, the analyte of interest is extracted from the sample matrix in an appropriate organic solvent and then injected either directly or after derivatization in a solvent such as hexane, methanol, or isooctane. This simple approach is not possible here because *tert*-butanol co-elutes with most organic solvents normally employed in GC. Gogineni et al.'s (4) solution to this problem was to employ a solvent with an unusually high boiling point, such as toluene, so that the solvent peak eluted after the analyte peaks. Our approach, on the other hand, was to simply eliminate the solvent altogether using SPME. This SPME technique will be useful in the GC analysis of any early-eluting component, including those commonly used as solvents in pharmaceutical and food preparations, such as hexane, acetone, ethyl ether, tetrahydrofuran, and ethyl acetate (7,10,11).

Because SPME is an equilibrium technique, analytes may not be completely extracted from the sample matrix (14). During the heating period, the analytes in the sample vial are partitioning between the three phases of the liquid sample, the headspace, and the SPME fiber. This creates two dependant equilibria that directly affect analyte recovery. Ideally, the SPME fiber would be exposed to the headspace for a sufficiently long time to ensure that equilibrium between all three phases had been reached; unfortunately, this rarely occurs in practice because reaching equilibrium may take anywhere from several minutes to hours depending on the analytes of interest, the fiber used, and the sample matrix (14).

In order to shorten the SPME sampling method, extraction can be ended before equilibration occurs as long as all variables that might affect recovery of analyte and reproducibility of the data, such as extraction and equilibration times, pH, and temperature, are held constant (14). Despite our efforts to control sampling parameters, in our standard curve, variation in the absolute area of the *tert*-butanol peak for each concentration was obvious. This variation in our replicate values (Table I) was undoubtedly due to irregularities in sampling parameters, such as slight variations in temperature, fiber exposure times, or equilibration times. We attempted to minimize this in several ways. For example, empty liposome material in buffer solution was used in our standard curve in an effort to control parameters associated with the sample matrix, such as pH because the actual liposome samples to be tested for *tert*-butanol were suspended in phosphate buffer. A blank of the buffer also failed to show any contaminants that might interfere with *tert*- or *sec*-butanol (Figure 1A). Equilibration, extraction, and desorption times were carefully controlled; however, a random 1°C fluctuation in temperature of the heating block was noted. This slight change in temperature throughout the 6 min equilibration and sampling period was the likely cause of the variation obtained in peak areas for replicate measurements. This again highlights the importance of using an internal standard with similar volatility and polarity as the analyte and performing SPME analysis in triplicate to account for slight changes in equilibrium. At least in this application, SPME is not a technique that lends itself to use of external standards.

Our standard curve was linear over a concentration range of ~10 to 600 µg/mL. This range was designed by considering the allowable limits for *tert*-butanol stipulated by the ICH Guidelines. There was no need to establish an upper bound on linearity because the maximum allowable *tert*-butanol level of 0.5% corresponded to 300 µg/mL with dilution in the 16 mL SPME vial. Amounts of *tert*-butanol above our linear region would clearly fail to meet the limits and, if necessary, could simply be quantified using serial dilutions to ensure that the analyte concentrations fall within the linear range established here. However, with our protocol, extending the lower limit is a more difficult problem to manage. Below 10 µg/mL, the response was no longer linear, due to insufficient headspace concentration and minimal absorption of the analytes on the SPME fiber. This is of course a direct result of our attempt to minimize sampling time by using a low temperature and short exposure time. A higher equilibration temperature would have undoubtedly produced a higher headspace concentration and, in turn, greater absorption of analytes on the SPME fiber. We chose a 30°C degree equilibration temperature because it was the lowest temperature that we could guarantee would always be above ambient. A higher temperature would have required a longer equilibration time, thereby extending the analysis time and defeating the purpose of this application.

Extending the period of time that the fiber was exposed to the headspace offered another approach to achieve greater recovery of the analytes. We made a preliminary investigation of this option by using an exposure time of 20 min, rather than 1 min, with a 312 µg/mL *tert*-butanol standard. This did result in a substantial increase in the peak areas of both *tert*- and *sec*-butanol but the longer exposure time also concentrated trace contaminants present in the *tert*- and *sec*-butanol (purity level of 99%), resulting in two contaminant peaks of a similar size as the analyte (Figure 1B). GC–mass spectrometry (MS) analysis of the *tert*- and *sec*-butanol standards gave a tentative identification of these components as *tert*-butyl isopropyl ether and di-*sec*-butyl ether and confirmed that the standards were the source of the contaminants. With a pre-concentration technique such as SPME, these additional volatiles will compete for active sites on the SPME fiber and if their levels varied, could create difficulties for quantification. However, with *tert*- and *sec*-butanol as sources of the contaminants, we know that their levels relative to the analytes will not vary. If the standard curves and samples are created using *sec*- and *tert*-butanol from the same source, then the levels of contaminants will remain the same in all material. Thus, the competition for active sites will also remain constant and the resulting decrease in analyte adsorption will be incorporated into the standard curves. Two additional peaks, partially co-eluting and fronting on *tert*- and *sec*-butanol, also appeared with the 20 min exposure time; however, they were not present with the 1 min exposure. In fact, with the short exposure time, GC–MS analysis was not able to detect these components, eliminating the concern that they were co-eluting with the analytes. Thus, for the purpose of developing a rapid method, a 1 min exposure time allowed us to quantify *tert*-butanol over a relevant range of concentrations and avoided the magnification of these contaminants.

A third modification that we considered was temperature programming. *tert* and *sec*-Butanol both elute soon after injection so that a total run time of 10 min with temperature programming was not strictly necessary. In fact, if elution of the analytes was the only consideration, a run time as short as 3 min would suffice (Figure 1C). However, the knowledge that components in addition to *tert*- and *sec*-butanol were present made us reluctant to end the run after only 3 min. We used the longer program to remove the late-eluting ether compounds and ensure that they would not appear in subsequent runs to potentially interfere with the determination of the components of interest. Regardless of total run time, data can be processed immediately after both *tert*- and *sec*-butanol have eluted, with no need to wait until the entire chromatogram is acquired. Thus, an effective time of analysis from placement of sample in vial to determination of peak area can be as little as 9 min. The slowest step in this process was the heating of the sample to 30°C. If the measurement was conducted in a climate-controlled facility, an equilibration temperature just slightly above ambient could be used, thereby decreasing time of analysis. In our facility, ambient temperature ranged from 20–28°C; to be prudent, an equilibration time based on the period of time required to raise a 16 mL sample from the lowest temperature in that range to 32°C was used.

To test this application, a sample of "empty" liposomes (composed only of cholesterol and phosphatidyl choline, prepared using *tert*-butanol as solvent) was provided by a local manufacturer. The sample was analyzed in triplicate and was found to contain a residual *tert*-butanol concentration of only 0.084 ± 0.009%, well below the 0.5% limit in the USP guidelines. As expected, the two ether contaminants eluting near 5 and 6 min and derived from *tert*- and *sec*-butanol were also present in the chromatogram (Figure 1D). The remainder of the chromatogram was free of potentially interfering peaks, demonstrating the applicability of this technique to actual liposome samples that have been carried through a typical manufacturing process.

Conclusions

We have presented a rapid SPME-GC–FID method for determination of trace chemicals in liposomes. Using our method, a single determination of *tert*-butanol, from time of sample receipt to final calculation, can be achieved in less than 9 min. We then used this method to determine *tert*-butanol levels in a liposome preparation provided by local industry, demonstrating it applicability to true process samples. We believe similar SPME methods, where speed of analysis, rather than recovery, is optimized will have wide applicability in monitoring residual solvent levels during processing.

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