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Gas chromatographic determination of residual levels of *tert.*-butanol from lyophilized liposomal formulations

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ABSTRACT

The use of liposomal formulations is rapidly gaining popularity in pharmaceutical research and development. Their preparation often involves the use of organic solvents such as *tert.*-butanol to dissolve lipophilic lipids. To improve the physicochemical stability of the liposomes, lyophilizing the product is one of the best available means. A gas chromatographic method for the determination of *tert.*-butanol residual levels in lyophilized liposomes, employing *sec.*-butanol as internal standard, using a flame ionization detector (FID) detector and a cross-linked dimethylpolysiloxane capillary column, was developed. The method described is simple, sensitive, rugged, reliable and reproducible and requires less time than other reported methods for the quantitation of *tert.*-butanol; it also has pharmaceutical applications.

INTRODUCTION

Nowadays, liposomes are increasingly being used as carriers for antigens and/or drugs for different routes of administration such as parenteral, pulmonary and nasal routes [1]. The physical stability of the liposomes during long-term storage has been a matter of intense investigation for quite some time. Several approaches have been developed to ensure stability of the liposomes, and lyophilization is one of the best methods available to achieve extended shelf-life for a liposomal product [2–4].

The freeze-drying of lipophilic lipids dissolved in an organic solvent, such as butanol, to form liposomes for parenteral administration purposes was reported as early as the 1970s [5]. It was proven that lipid solubility is four to five times greater in *tert.*-butanol than in other organic solvents such as ethanol [6,7] and, therefore, the use of *tert.*-butanol as solvent to dissolve the lipids in the preparation of lyophilized liposomes is becoming more popular [4,5]. Whenever an organic solvent is used in the preparation of a lyophilized formulation, it is practically quite difficult to remove the solvent completely during the lyophilization process, and therefore the traces or residuals of the solvent remaining in the product must be detected and quantitated as required by the regulatory authorities [1,8].

Because of its unsurpassed resolving power, speed, sensitivity and small sample requirements, gas chromatography is one of the most widely used techniques in analytical chemistry for analysing traces and very low residuals of volatiles such as organic solvents. The reported GC techniques used to monitor the residual alcohol contents from drug raw materials [9] and biological fluids [10,11] were found to be too complex and

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time-consuming to be utilized for liposomal drug formulations. Hence, the authors have developed a simple, reliable and reproducible gas chromatographic method to quantitate the residual levels of *tert.*-butanol from freeze-dried liposomal formulations.

EXPERIMENTAL

Reagents

The analyte *tert.*-butanol, lot 920454, and the internal standard *sec.*-butanol, lot 920838, both certified grade, were obtained from Fisher Scientific (Fairlawn, NJ, USA). Toluene, used as an extraction solvent (lot BE505), was purchased from Burdick & Jackson, a division of Baxter Healthcare (Muskegon, MI, USA). During the solvent evaluation and selection stage, toluene from two other sources [99% grade, lot 02127PY, from Aldrich (Milwaukee, WI, USA) and Baker Resi-Analyzed grade, lot 503724, from J. T. Baker (Philipsburg, NJ, USA)] was also used. Several lots of lyophilized liposomes prepared from a proprietary mixture in our laboratory were analysed for the residual levels of *tert.*-butanol.

Instrumentation

The GC workstation consisted of Varian 3500 gas chromatograph (Varian Assoc., Sunnyvale, CA, USA) equipped with flame ionization detector and 1077 split/splitless capillary injector with automatic injection start signal actuator. Maximum operating temperature of both detector and injector was 420°C. The system utilizes an inboard data handling (IBDH)-type plotter. The gas chromatograph unit was equipped with a RT_x-1 (obtained from Restek Corporation, Bellefonte, PA, USA) 100% cross-linked dimethylpolysiloxane fused-capillary column, lot 8878A, [30 m × 0.25 mm I.D., 0.10 μm film thickness (d_f value)]. Ultra-pure carrier-grade compressed helium (Air Products, Middletown, OH, USA) was used as carrier gas.

Experimental conditions

The temperature of the injector was maintained at 280°C and that of the detector at 275°C. Initial column temperature was determined taking into account the highly volatile nature of the isomeric butanols and was set at 40°C. After 2 min at 40°C, the temperature of the oven (column) was programmed to rise to 160°C (100°C for the standard solutions since these do not contain any lipids) at a rate of 30°C/min with a 0.5 min hold time at this high temperature to flush out the lipids from the liposomes. The head pressure and its dependent parameters, *i.e.* flow-rate and linear velocity, have a direct effect on the degree of sharpness and resolution of the individual peaks of interest. To quantitate both high and low levels of *tert.*-butanol from in-process as well as final samples, it is necessary to use different head pressures (and, consequently, different flow-rate and linear velocity values) with different split ratios to maintain the effectiveness of the methods in achieving good separation and good resolution. While the head pressure was set at $5.17 \cdot 10^{-1}$ bar (0.6 ml/min flow-rate and 15.2 cm/s linear velocity for a split ratio of 350:1), it was adjusted to $7.38 \cdot 10^{-1}$ bar (0.9 ml/min flow-rate and 22.3 cm/s linear velocity) when using the 75:1 split ratio.

Methods

Calibration curve. Standard solutions of various concentrations of *tert.*-butanol containing *sec.*-butanol as an internal standard were prepared as follows:

(a) A stock solution of the solvent, toluene, containing a known amount of the internal standard was prepared.

(b) The required quantity of the stock solution was accurately weighed into a clean, dry and stoppered glass vial.

(c) The necessary quantity of *tert.*-butanol was added directly to the stock solution in the vial and the weight of *tert.*-butanol added was calculated.

The concentration of the internal standard in the stock solutions used for generating the calibration curve with split ratios of 75:1 and 350:1 was 30 mg/kg and 250 mg/kg (0.003%, w/w, and 0.025%, w/w), respectively. Split ratios of 350:1 and 75:1 were used for high and low *tert.*-butanol concentrations, respectively.

Extractability. The extractability of the internal standard from the sample solution in the presence of the lyophilized cake was established by using 250 ± 20 mg/kg (0.025 ± 0.002%, w/w) *sec.*-butanol. The effect on recovery of spiking the dry, lyophilized sample cake directly with internal standard compared with spiking the resultant mixture after the dissolution of the dry sample cake in the solvent was studied. The extractability of *sec.*-butanol was established by comparing the peak areas from a sample solution containing both the solvent and the lyophilized cake of the sample and, in addition, a known quantity (170 ± 20 mg/kg; 0.017 ± 0.002%, w/w) of *tert.*-butanol with that of a standard solution containing 190 mg/kg (0.019%, w/w) *tert.*-butanol in toluene after performing the necessary baseline corrections.

Sample preparation. The stock solution containing 300 ± 100 mg/kg (0.03 ± 0.01%, w/w) internal standard was used to establish extraction efficiency, and to optimize the sample (lyophilized liposomal cake) size requirement. Sample solutions were prepared by adding a known quantity of the stock solution containing internal standard to the accurately weighed lyophilized sample cake followed by sonication for *ca.* 2 min to ensure complete dissolution/dispersion of the solid sample cake in the solvent before analysing. Residual levels of *tert.*-butanol were quantitated by extracting it from varying amounts (approximately from *ca.* 100 mg/kg to $3.0 \cdot 10^4$ mg/kg or 0.01%, w/w, to 3.0%, w/w) of sample cake into a series of stock solutions.

General precautions taken. The concentrations of the analyte and the internal standard were always expressed as weight by weight [and/or per cent weight by weight (%, w/w)] for accurate results. The vials containing the sample/standard solutions were filled to 95% of their capacity and stoppered immediately following the addition of the solvent/analyte/internal standard to minimize the evaporation of the volatile butanols and the extraction solvent. Chromatographic profiles of toluene obtained from different sources were evaluated before approving Burdick and Jackson as the source on the basis of interfering impurity peaks. Accurately measured (1.0 ± 0.25 μl) samples were injected manually into the preheated injector with the aid of a Hamilton microsyringe (10 μl capacity). Chromatograms of the extraction solvent, toluene, were taken prior to injecting samples for the identification and proper separation of impurity peaks from those of interest, *i.e. tert.*-butanol and *sec.*-butanol.

RESULTS AND DISCUSSION

It has been reported that the selection of solvent plays a key role in developing a gas chromatographic method and that the higher the boiling point of the solvent the longer are the retention times of the solutes eluted near the solvent peak. It has also been proven that peak shape and resolution-related problems will occur for the solute peaks eluting after the solvent if the solvent front is not utilized [12]. The relatively high volatility and low boiling points of the analyte, *tert.*-butanol and the internal standard, *sec.*-butanol, result in faster elution at low temperatures. Since toluene has a high boiling point compared with other homologous organic solvents with 1–8 carbon atoms and has a longer retention time, it was selected as the solvent. With solutes eluting much before the solvent, the problems associated with the solvent front and the effect of the solvent's boiling point on the retention times of the solutes of interest were completely avoided, which resulted in a relatively short (less than 7 min) analysis time.

Typical chromatographic profiles of toluene (Fig. 1) show some impurity peaks. It was observed that the concentration of these impurities affects the separation and the resolution of the solute peaks of interest, which elute just before the impurity peaks. The effect of these impurities on the separation and resolution of solute peaks is more significant when a sample is analysed with a split ratio of 75:1. Therefore, toluene samples obtained from different sources were evaluated and the one with lowest impurity interference (source: Burdick & Jackson) with the peaks of the analyte and the internal standard was used in the subsequent studies.

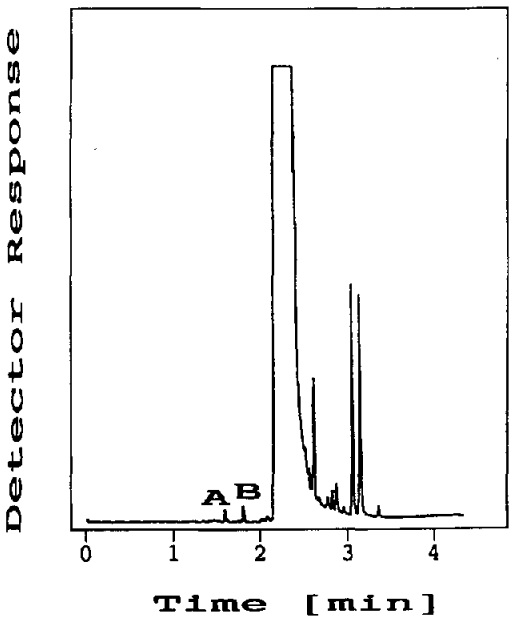

Fig. 1. Chromatogram of the extraction solvent, toluene, with impurity peaks (A) and (B). Conditions: injector, 280°C; detector, 275°C; column, 40°C with 2 min hold time; rate of heating, 30°C/min; column final temperature, 100°C with 0.5 min hold time; split ratio, 350:1; RT_x-1 dimethylpolysiloxane capillary column (30 m × 0.25 mm I.D., 0.1 d_f).

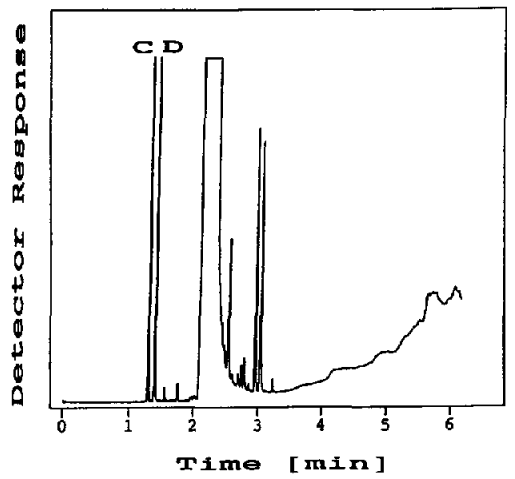

Fig. 2. Chromatogram of an extract of a lyophilized sample showing *tert.*-butanol (C) and *sec.*-butanol (D). Conditions: same as in Fig. 1, except for final temperature of the column, which was set to 160°C with 0.5 min hold time (run time was 6.5 min). Retention times: 1.30 min for *tert.*-butanol and 1.41 min for *sec.*-butanol.

Fig. 2 illustrates a typical chromatogram resulting from the analysis of an extract of lyophilized sample containing *sec.*-butanol and shows the effectiveness of the method in achieving good separation between the isomeric butanol peaks and good resolution among the solute and solvent peaks. The high peak-height values obtained for the butanols indicate the degree of sharpness and resolution achieved for the individual peaks of interest.

The standard curve obtained with a 350:1 split ratio showed good linearity of peak areas and peak heights with increasing concentrations of *tert.*-butanol ranging from 20 ppm to 11 000 ppm (20 mg/kg to 11 000 mg/kg), which is a good indication of the method's effectiveness, and shows that the detector response is independent of analyte concentration. This linearity is further confirmed by the peak area ratios of the peaks of *tert.*-butanol and *sec.*-butanol (r = 0.9999).

With very few minor parameter adjustments such as head pressure and split ratio, this method has effectively been used to quantitate, with good accuracy, *tert.*-butanol levels as low as 1 ppm. The retention times of both the butanols decreased from 1.30 ± 0.10 min to 0.90 ± 0.01 min for *tert.*-butanol and from 1.40 ± 0.10 min to 0.99 ± 0.01 min for *sec.*-butanol when the split ratio was decreased from 75:1 to 350:1 due to the changes in the experimental conditions. The standard curve yields a correlation coefficient value (r) of 0.9963 for the peak area ratio of *tert.*-butanol and *sec.*-butanol peaks. Thus, this method affords a linear response within the 3 log concentration range, *i.e.* from 1 ppm to slightly more than 1000 ppm (1 mg/kg to 1000 mg/kg) of *tert.*-butanol. The mean and standard deviation values of both calibration curves and sample solutions were calculated from multiple (n = 6) injections.

In order to establish the optimum concentration range of the sample to be used for accurate quantitation of the solvent residual levels in the lyophilized samples, *tert.*-butanol was extracted from varying quantities of the sample cake with toluene and was analysed directly. Results are tabulated in Table I.

The mean and the standard deviation of the

TABLE I

RESIDUAL LEVELS OF *tert.*-BUTANOL RECOVERED FROM LYOPHILIZED SAMPLE CAKE

Sample weight (%, w/w)	*tert.*-Butanol content (mean ± S.D., $n=6$) (%, w/w)	Coefficient of variation (%)
0.0165	Not detected	–
0.0468	Not detected	–
0.1150	0.756 ± 0.058	7.7
0.2355	0.668 ± 0.096	14.4
0.5504	0.706 ± 0.048	6.8
1.0775	0.756 ± 0.055	7.3
1.7015	0.883 ± 0.074	8.3
2.7620	0.881 ± 0.047	5.3

recovered *tert.*-butanol (n = 6, 7800 ± 900 mg/kg or 0.78 ± 0.09%, w/w), indicate that the recovery is fairly constant and is independent of the sample concentration in the range 1000 mg/kg to approximately $3.0 \cdot 10^4$ mg/kg or 0.1% (w/w) to *ca.* 3.0% (w/w). This affords an opportunity to decide on the sample size, depending on the availability of the sample, yet obtain accurate values of the *tert.*-butanol residuals in the sample. Since the sample vial normally contained about 1 g of the lyophilized liposomal cake, sample size is not a critical factor and, therefore, a relatively high sample size (about $1 \cdot 10^4$ mg/kg or 1%, w/w) was used with a split ratio of 350:1 to minimize the errors associated with smaller quantities of samples.

An additional advantage of using the high split ratio is that the in-process samples, which normally contain a high *tert.*-butanol content, as well as the final lyophilized samples with low residue levels, can be analysed using the same experimental conditions, to keep the experimental variations to a minimum. As mentioned earlier, a very good linearity was achieved [correlation coefficient (r) of 0.9996 for the peak-area ratio of *tert.*-butanol and *sec.*-butanol peaks] with increasing concentration of *tert.*-butanol, which increases with increasing mass of the sample cake.

CONCLUSIONS

A gas chromatographic analytical method has been developed as a simple, sensitive, reliable and reproducible way of analysing lyophilized liposomal preparations of proprietary mixtures for very low residual levels of *tert.*-butanol with very good accuracy and precision. Since a sample can be analyzed in less than 7 min, this method should be highly useful in analysing the in-process samples of liposomal formulations within a short period. This method, with a small split ratio, can also be used for quantitating lower levels of *tert.*-butanol residuals from a small quantity of analyte sample.

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