TECHNICAL NOTE

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Gas Chromatographic–Mass Spectrometric Identification and Quantitation of Benzyl Alcohol from Human Serum and Postmortem Blood after Derivatization with 4-Carbethoxy Hexafluorobutyryl Chloride: A Novel Derivative

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ABSTRACT: Benzyl alcohol is commonly used as an antibacterial agent in a variety of pharmaceutical formulations. Several fatalities in neonates have been linked to benzyl alcohol poisoning. Most methods for measuring benzyl alcohol concentrations in serum utilize direct extraction followed by high performance liquid chromatography. We describe a novel derivatization of benzyl alcohol using 4-carbethoxyhexafluorobutyryl chloride after extraction from human plasma, and subsequent analysis by gas chromatography-mass spectrometry (GC/MS). The derivative was eluted at a significantly higher temperature and the method was free from interferences from more volatile components in serum and hemolyzed specimens. However, with postmortem specimens, we observed multiple peaks which were eluted at a very high temperature, long after derivatized benzyl alcohol and the internal standard. Therefore, baking the column at 310°C is recommended after analysis of a postmortem specimen. Another advantage of this derivatization technique is the conversion of low molecular weight benzyl alcohol (MW 108) to a high molecular weight derivative (MW 358). The positive identification of benzyl alcohol can be easily achieved by observing a distinct molecular ion at m/z 358 as well as other characteristic ions at m/z 107 and 91. Quantitation of benzyl alcohol in human serum can easily be achieved by using 3,4-dimethylphenol as an internal standard. The within run and between run precisions (using serum standard of benzyl alcohol: 50 mg/L) were 2.2% ($\bar{X}$ = 50.6, SD = 1.1 mg/L), and 6.9% ($\bar{X}$ = 50.8, SD = 3.5 mg/L). The assay was linear for the serum benzyl alcohol concentrations of 5 mg/L to 200 mg/L and the detection limit was 1 mg/L. We observed no carry-over problem in our assay as when 2 μL ethyl acetate was injected into the GC/MS after analyzing serum specimens containing 200 mg/L of benzyl alcohol, we observed no peak for either benzyl alcohol or the internal standard in the total ion chromatogram.

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Benzyl alcohol at concentrations of 0.9–2.0% is commonly used as an antibacterial agent in many pharmaceutical formulations especially intended for intravenous administration. Intraventricular hemorrhage and death in preterm neonates has been associated with the use of fluid containing benzyl alcohol. Exposure to benzyl alcohol was significantly associated with the development of kernicterus (1). Benzyl alcohol preservatives in intravascular flash solutions has been reported to cause neurological deterioration and death in low birth weight infants (2). Brown et al. also reported symptoms of neurological deterioration, hematologic abnormalities, severe metabolic acidosis, respiratory distress, hepatic and renal failure, hypotension and cardiovascular collapse from benzyl alcohol intoxication in neonates (3). Garshanik et al. reported blood benzyl alcohol concentrations ranging from 66 mg/L to 148 mg/L in six infants with respiratory distress resulting from benzyl alcohol toxicity (4). Deaths in immature animals have been reported after oral and intravenous administration of Ringer's solution containing 1.5% benzyl alcohol (5). Benzyl alcohol is widely used in organic synthesis and as a solvent for various compounds, for example cellulose.

Different methods have been reported in the literature for identification and quantitation of benzyl alcohol, but most of the techniques utilize high performance liquid chromatography (HPLC) and the identification of benzyl alcohol is solely based on the retention time. Tan et al. reported a HPLC method for determination of benzyl alcohol using a C_{18} column and water-acetonitrile-glacial acetic acid as a mobile phase (6). Block and Levine reported a qualitative method for the detection of benzyl alcohol, benzoic acid, and nitrobenzene in benzaldehyde USP specimens by a normal phase HPLC (7). Rego and Nelson reported simultaneous determination of hydrocortisone and benzyl alcohol in pharmaceutical formulation using reverse phase HPLC (8).

Czarny and Hornbeck reported a novel derivatization of amphetamine and methamphetamine using 4-carbethoxyhexafluorobutyryl chloride (9). Because benzyl alcohol has a free hydroxyl group, 4-carbethoxyhexafluorobutyryl chloride also can potentially

react with benzyl alcohol. The advantage is the significantly higher molecular weight of the derivative (benzyl alcohol, MW:108, derivative, MW:358). Therefore, a positive identification by mass spectrometry could be enhanced by this process which should certainly be helpful in medical-legal cases. Here we report a new derivatization of benzyl alcohol after extraction from human serum and postmortem blood using 4-carbethoxyhexafluorobutyryl chloride and the subsequent analysis by GC/MS.

Materials and Methods

Reagents

Benzyl alcohol and the internal standard 3,4-dimethylphenol were purchased from Aldrich Chemical Company (Milwaukee, WI). The derivatizing agent 4-carbethoxyhexafluorobutyryl chloride was obtained from PCR chemicals (Gainsville, FL).

Instrumental Conditions

The GC/MS analysis was performed by a Model 5890 gas chromatograph coupled with a 5972 series mass selective detector (Hewlett Packard, Palo Alto, CA). The capillary column (Ultra-1, 25 m by 0.2 mm) also obtained from Hewlett Packard was coated with cross linked methyl silicone (0.33 μm film thickness). The initial oven temperature of the gas chromatograph was 140°C. After maintaining that temperature for 2 min, the oven temperature was increased at a rate of 10°C/min to an oven temperature of 220°C. Then the oven temperature was increased at a rate of 15°C/min to reach a final oven temperature of 280°C. The final oven temperature was maintained for another 3 min in order to clean the column. The run time was 17 min. The mass spectrometer was operated in the electron ionization mode (scan m/z 50–500). For analysis of postmortem specimens, we baked the column at 310°C for an additional 10 min prior to the next injection. This baking process is helpful in avoiding interfering peaks in the next injection.

Standards and Controls

A stock solution of 3,4-dimethylphenol, the internal standard (10 mg/mL) was prepared in chloroform. One stock solution of benzyl alcohol in water (for supplementing serum with benzyl alcohol) and a separate stock solution of benzyl alcohol in chloroform (for the recovery studies) were prepared. Different aliquot of pooled serum was supplemented with various concentrations of benzyl alcohol for our study. The serum pools were prepared from left over plasma obtained from the local blood bank. Expired blood was obtained from the local blood bank. We also pooled several postmortem blood which was saved for two years and then discarded by the office of the medical examiner.

Sample Preparation

To 2-mL aliquot of serum, hemolyzed blood or postmortem blood (supplemented with benzyl alcohol), 10 μL of the internal standard stock solution was added. After that, 10 mL of chloroform was added to extract benzyl alcohol along with the internal standard from the serum. After vortex-mixing for 1 min, samples were mixed for an additional 10 min in a rotary mixture. The upper aqueous layer was separated from the lower chloroform layer by centrifugation at 1500 g for 10 min. Then the upper aqueous layer was discarded, and the lower organic layer was carefully transferred to a conical test tube. The chloroform extract was evaporated to dryness using air at room temperature, and 50 μL of 4-carbethoxyhexafluorobutyryl chloride was added to the dry extract. After incubation at 85°C for 25 min, the excess derivatizing agent was evaporated, and the residue was reconstituted with 100 μL of ethyl acetate. Then 1–2 μL was injected into the GC/MS. The quantitation of the benzyl alcohol peak was done by comparing the area under the curve with the area under the curve for the internal standard which eluted after the derivatized benzyl alcohol peak.

Results and Discussion

Chromatographic Properties of Derivatized Benzyl Alcohol

We observed excellent chromatographic properties of both derivatized benzyl alcohol and the internal standard, 3,4-dimethylphenol. The two peaks were well separated with the retention time of derivatized benzyl alcohol being 10.3 min and the retention time of derivatized internal standard being 11.5 min. We used a relatively long solvent delay (8.5 min) when the mass spectrometer was off in order to obtain a clean chromatogram. We also observed no interfering peaks in our chromatogram when grossly hemolyzed specimens were supplemented with benzyl alcohol. However, with pooled postmortem specimens, which were prepared from two years old discarded blood, we observed multiple peaks which were eluted at a very high temperature. Those peaks showed substantially longer retention times than the derivatized benzyl alcohol and the internal standard and did not interfere with the analysis. Therefore, we concluded that postmortem blood also can be successfully analyzed for the presence of benzyl alcohol (Fig. 1).

In order to further investigate the matrix effect from hemolyzed blood and postmortem specimens, we supplemented them with various concentrations of benzyl alcohol and compared the observed benzyl alcohol concentrations with the target concentrations. In all cases, we observed comparable values indicating that our assay can be used for identification and quantitation of benzyl alcohol in grossly hemolyzed as well as postmortem specimens (Table 1).

Mass Spectral Characteristics of Derivatized Benzyl Alcohol

Benzyl alcohol is a small molecule with a molecular weight of 108. In our new derivatization protocol, the molecular weight of 4-carbethoxyhexafluorobutyryl derivative of benzyl alcohol was 358. We observed a distinct molecular ion at m/z 358 (relative abundance 15.8%) in the mass spectrum of the 4-carbethoxyhexafluorobutyryl derivative of benzyl alcohol. Another characteristic peak was observed at m/z 107 (relative abundance: 14.8%) due to the loss of 4-carbethoxyhexafluorobutyryl moiety. The base peak was observed at m/z 91 because of the stability of $C_6H_5CH_2+$ ion (Fig. 2).

The 4-carbethoxyhexafluorobutyryl derivative of 3,4-dimethylphenol, the internal standard, showed a strong molecular ion at m/z 372 (relative abundance: 59.4%). The base peak was observed at m/z 122 again due to the loss of 4-carbethoxyhexafluorobutyryl moiety. Other strong peaks were observed at m/z 195, 91, and 77 (Fig. 3).

Precision, Linearity and Detection Limit

The within run and between run precision of the assay were determined by analyzing a serum pool supplemented with benzyl alcohol to achieve a final concentration of 50 mg/L. The within run C.V. was 2.2% ($\bar{X}$ = 50.6, SD = 1.1, n = 10), whereas the between run C.V. was 6.9% ($\bar{X}$ = 50.8, SD = 3.5, n = 7). The

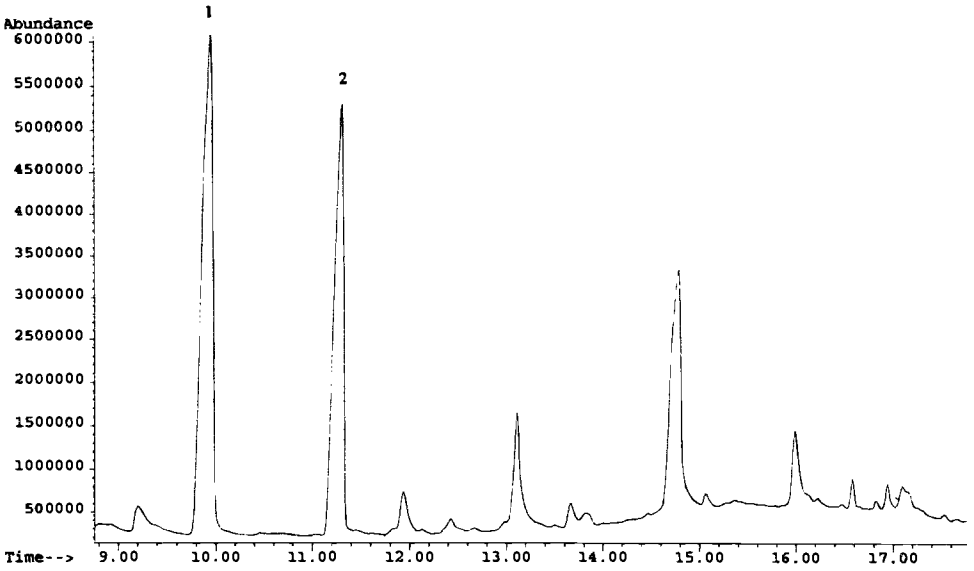

FIG. 1—*Total ion chromatogram of a typical postmortem blood extract showing the separation between benzyl alcohol and the internal standard after derivatization with 4-carbethoxyhexafluorobutyryl chloride. The peak 1 is the derivatized benzyl alcohol and the peak 2 is the derivatized internal standard. The concentration of benzyl alcohol was 60 mg/L.*

TABLE 1—*Observed and target benzyl alcohol concentrations in grossly hemolyzed and postmortem specimens.*

Specimen	Benzyl Alcohol, mg/L	
	Target	Observed
Gross hemolysis	60.0	63.7
Gross hemolysis	25.0	24.2
Postmortem	80.0	86.8
Postmortem	60.0	64.5
Postmortem	50.0	45.6
Postmortem	25.0	23.9
Postmortem	25.0	27.6

Postmortem specimens were two years old.

assay was linear for a serum benzyl alcohol concentration between 5 mg/L and 200 mg/L. Using X-axis as the target concentration and Y-axis as the observed concentration in the linearity study, we obtained the following regression equation.

$$y = 1.07 \times + 2.4, (r = 0.99).$$

The detection limit was 1 mg/L serum benzyl alcohol concentration. The detection limit can be further lowered by reconstituting the residue after derivatization in 50 μL of ethyl acetate instead of 100 μL of ethyl acetate which is our standard protocol.

Recovery Studies

For the recovery study, we made another standard solution of benzyl alcohol in chloroform. The recovery studies were performed

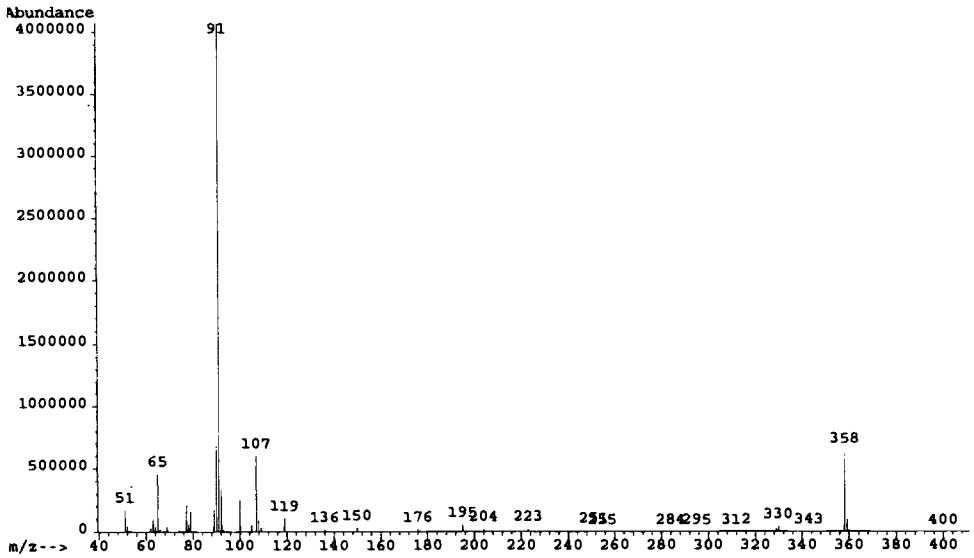

FIG. 2—*Mass spectrum of 4-carbethoxyhexafluorobutyryl derivatives of benzyl alcohol.*

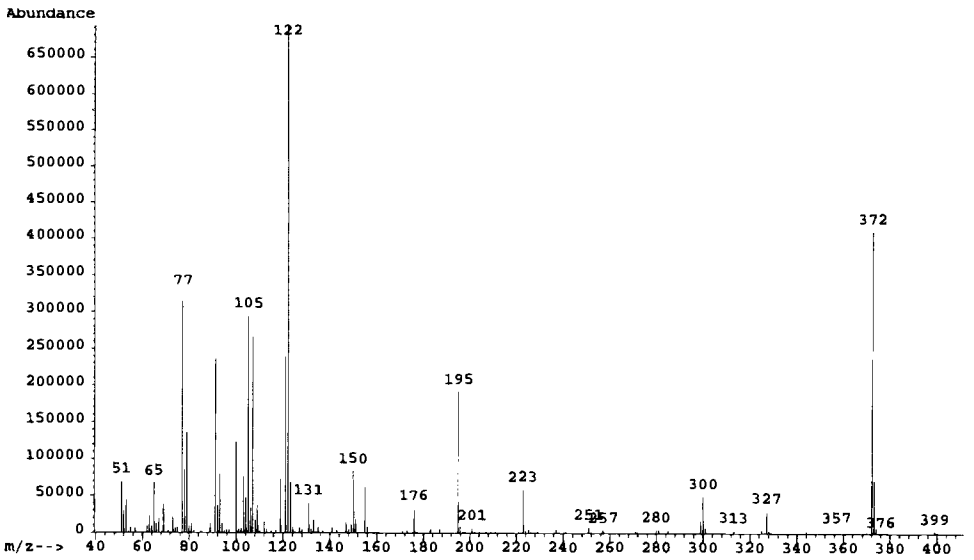

FIG. 3—*Mass spectrum of 4-carbethoxyhexafluorobutyryl derivative of 3,4-dimethylphenol, the internal standard.*

by comparing the peak area of the benzyl alcohol standards without extraction and after extraction from serum. For supplementing serum with benzyl alcohol, we again used the aqueous stock solution of benzyl alcohol. The average recovery of benzyl alcohol at a serum concentration of 50 mg/L was 87.3% and at a concentration of 200 mg/L was 84.3%. The average recovery of 3,4-dimethylphenol, the internal standard was 78.8% at the concentration used for the entire study (50 mg/L).

Carry-Over

In order to study any carry over problem, we analyzed a serum specimen supplemented with benzyl alcohol to achieve a final concentration of 200 mg/L. Immediately after the GC/MS run, we injected 2 μL of ethyl acetate into the GC/MS. We observed no peak for either derivatized benzyl alcohol or the internal standard. We performed our analysis in triplicate. As expected, we did not observe any peaks with our blank injection after analyzing another serum specimen supplemented with 150 mg/L of benzyl alcohol (duplicate measurement). We also observed no carry over problem after analysis of hemolyzed specimens. However, with one occasion with postmortem blood, we observed two peaks which were eluted at a very high temperature (no interfering peaks for benzyl alcohol or internal standard). The problem can be easily circumvented if the column was baked for 10 min at 310°C. Therefore, we concluded that the GC/MS assay for derivatized benzyl alcohol does not have any carry over problem.

Application of the Assay

Investigation of death from benzyl alcohol toxicity would certainly involve determination of benzyl alcohol concentration in blood. The positive identification of benzyl alcohol is also very important in medico-legal cases. Mass spectrometric identification is considered the gold standard for drugs. The earlier reports on quantitation of benzyl alcohol in blood almost always use a HPLC method without any mass spectrometric analysis. Therefore, identification of benzyl alcohol is solely done based on the retention time and there is a possibility of a misidentification of a compound with a retention time close to that of benzyl alcohol. In our novel derivatization technique, benzyl alcohol is identified based on the distinct molecular ion at m/z 358 as well as other characteristic secondary ions in addition to the correct retention time. Moreover, the 4-carbethoxyhexafluorobutyryl derivative of benzyl alcohol was eluted at a relatively higher oven temperature and the assay was free from interferences from other volatile compounds in serum. Our protocol utilizes a simple extraction using chloroform and a simple derivatization technique and the assay can easily be adopted in a toxicology laboratory.

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