Tri-Butoxyethyl Phosphate as a Contaminant in B-D Vacutainers

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An unknown compound was extracted from serum samples. The compound was studied by gas-liquid chromatography, thin-layer chromatography, and mass spectrometry, and was positively identified as tri-butoxyethyl phosphate. The source of this compound was traced to B-D Vacutainers used to collect blood. The importance of this finding is briefly pointed out.

During studies with gas-liquid chromatography (GLC) to detect compounds in the serum of patients with parasitic infections, we found a compound in some sera which was not present in the sera from healthy controls. This paper reports how this compound was studied and identified as a contaminant in the B-D Vacutainers.

MATERIALS AND METHODS

Extraction of unknown. The unknown compound could be extracted from serum samples, and later from the empty B-D vacutainers, with most lipid solvents such as ethyl alcohol, ethyl acetate, benzene, hexane, etc. Addition of acid enhances the extraction. A few drops of 4% HCl were added routinely to a 0.5- to 1-ml amount of serum. Serum was partially dried and extracted with 10 ml of ethanol. Ethanol extract was dried, and the residue was extracted with ethyl acetate. Then the volume of solvent was reduced to 0.1 ml prior to injection into the gas chromatograph.

Formation of derivatives. Two methylation procedures were used. In the first method, boron fluoride (10% in methanol, Eastman Kodak Co.) was used. The reagent was added to the sample in a sealed test tube and left in boiling water for 5 min. After cooling, water was added and the mixture was extracted with petroleum ether. Petroleum ether was dried with anhydrous sodium sulfate, and its volume was reduced (to 0.1 ml) under a gentle flow of nitrogen prior to GLC. In the second method of methylation (2), methanolic HCl was used, as described earlier.

Silylation was accomplished by the use of hexamethyl-disilazane and trimethylchlorosilane (Applied Science Labs, State College, Pa.), with pyridine as a catalyst according to the procedure reported elsewhere (3).

Trifluoroacetic anhydride (TFA) derivatives were prepared by adding ethyl acetate and TFA (Eastman Kodak Co.) to the samples and leaving them at 70 C for 1 h. After the samples were cooled, they were evaporated to dryness, and the residues were dissolved in 0.1 ml of ethyl acetate before GLC analysis. All the solvents used in this study were reagent grade.

GLC. GLC was carried out on a Perkin-Elmer model 990 instrument with flame ionization detectors. Three glass columns (6 ft by 0.25 inch outside diameter [182.88 by 0.635 cm]) were used. These columns were packed with 3% OV-1 on Gas-Chrom Q 100 to 200 mesh, 3% OV-17 on Gas-Chrom Q 100 to 120 mesh, and 3% QF1 on Gas-Chrom Q 100 to 120 mesh. Instrument parameters were: injection port temperature, 265 C; detector temperature, 285 C; and column temperature, 220 C. Helium was used as the carrier gas with an inlet pressure of 30 psi.

Thin-layer chromatography (TLC). TLC plates (20 by 20 cm) precoated with 0.25 mm of Silica Gel G (Analtech, Inc., Newark, Del.) were used. The plates were developed and sprayed with phosphomolybdic acid and 2',7'-dichlorofluorescein as reported earlier (1). In addition, some plates were sprayed with sulfuric and phosphoric acid reagents prepared according to Stehl (5).

Mass spectrometry. Mass spectrometry was done by using a gas chromatography/mass spectrometry LKB 9000 instrument. The mass spectra were recorded at an electron energy of 70 eV and a trap current of 60 μA, ion source temperature of 290 C, and a molecular-separator temperature of 250 C. The accelerating voltage was 3.5 kV. The GC column temperature was 230 C. This column was 10 ft (304.8 cm) long and was packed with 3% SE-30 on 80 to 100 mesh Supelcoport (Supelco Inc., Pa.).

RESULTS AND DISCUSSION

Several chemical tests were performed on the unknown compound. Two methylation procedures followed by GLC did not change the retention time of the unknown peak, which indicates that the compound did not contain a carboxyl group. Silylation and subjection of the sample to formation of TFA derivatives also failed to alter the retention time of the unknown peak. The former indicated a lack of hydroxyl group, and the latter indicated the absence of amine groups in the molecule.

TLC of the unknown along with some known
methyl esters and triglycerides showed that the unknown compound migrated at the same rate as lower triglycerides. Furthermore, staining characteristics of the unknown compound were similar to those of lower triglycerides. GLC showed that retention time of the unknown peak was higher than tricaprin and lower than tricaprylin. Moreover, the unknown compound did not release any fatty acid after saponification and subsequent acidification.

Information obtained by studying the mass spectrum of the unknown compound plus information obtained by GLC and TLC made it possible to identify the unknown peak as tributoxyethyl phosphate (TBEP), with the chemical structure shown in Fig. 1.

The mass spectrum of the unknown compound is shown in Fig. 2. A peak of electronic mass (m/e) 398 due to the molecular ion could be seen in the spectrum, but its intensity was very low. The base peak of the spectrum is at m/e 57 which is due to \([\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{-O-CH}_2\text{-CH}_2\text{-P=O}}\) and alpha cleavages. A peak of m/e 355 is due to the loss of \([\text{CH}_3\text{CH}_2\text{CH}_2\text{-CH}_2\text{-O}}\) and beta cleavage from the molecular ion. The spectrum also shows hydrocarbon-type peaks with the general formula of \(\text{C}_n\text{H}_{2n}\), which are accompanied by smaller peaks 1, 2, and 3 mass units lower and the \(^{13}\text{C}\) "isotope" peak 1 mass unit higher. The peak at m/e 45 could be due to hydrogen rearrangement. Peaks at m/e 43, 57, 71, 85, 99, etc. are due to \([\text{C}_n\text{H}_{2n-1}\text{O}}\) series, and peaks at m/e 44, 58, 72, 86, 100, etc. are due to \([\text{C}_n\text{H}_{2n}+\text{O}}\), series contribute to the peaks at m/e 45, 59, 73, 87, 101, etc. All of this information is consistent with an ether-type compound (4). The significant peak at m/e 299 is due to M-99 (99 corresponds to the loss of one of the chains in the molecule minus one molecule of water). Subsequent losses of several methylene groups \([\text{CH}_2\text{-O}}\) and oxygen from the remainder of the molecule (299) will result in peaks such as 271, 255, 227, and 199, etc. in the spectrum.

TBEP was obtained from Pfaltz & Bauer, Inc.

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**Fig. 1.** Chemical structure of tri-butoxyethyl phosphate.

**Fig. 2.** Mass spectrum of unknown compound, later identified as TBEP.
Flushing, N.Y. The retention time of TBEP matched on three different columns in GLC. The mass spectrum of TBEP was identical to the spectrum of the unknown. Mass spectral data were confirmed by using the computer matching system of DCRT/CIS of National Institutes of Health, Bethesda, Md.

Further study revealed that TBEP was a serum contaminant whose source was the B-D Vacutainer. This was confirmed by examining the gas chromatogram of a serum sample before it was transferred to a B-D Vacutainer. The unknown peak was absent in the chromatogram before the sample was transferred to the vacutainer, but present after the sample had been in the vacutainers for a short period of time. TBEP could be extracted from B-D Vacutainers with red, gray, lavender, pink, and green stoppers; however, the quantity of TBEP in vacutainers with green stoppers was much less than the others. TBEP was present in all sizes of B-D Vacutainers with red stoppers, and the quantity appeared to be proportional to the size of the vacutainer. In a study of an unused 15-ml B-D Vacutainer, approximately 300 µg of TBEP was extracted from the glass wall and inside portion of the stopper. Only about 30 µg of TBEP was obtained from the glass wall. This compound was absent from all sera that were drawn by syringe and needle. The use of vacutainers by hospitals and private physicians is a common practice. Because TBEP could be extracted from serum with lipid solvents and has characteristics similar to triglycerides (on TLC plates), its presence may cause a problem when serum lipids are being studied. The presence of TBEP may also affect phosphorus determination. Various institutions such as the Center for Disease Control (Atlanta, Ga.) routinely receive blood, serum, urine, and other clinical specimens for testing. These specimens are routed to the appropriate laboratories within the institution for bacteriological, mycological, parasitological, virological, biochemical and immunological tests. Some of these clinical samples are either sent in Vacutainers or are originally collected in Vacutainers and later transferred to other containers. Therefore, studies should be made to compare and determine the microbiological, biochemical, and immunological properties of clinical specimens contaminated with TBEP.

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