GLC Analysis of Bis(2-ethylhexyl) Phthalate Plasticizer in Tissue and Plasma

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Abstract □ A method for the GLC analysis of the plasticizer, bis(2-ethylhexyl) phthalate, in lung, liver, spleen, and plasma in levels as low as 1 mg % is described. The method employs an internal standard, bis(*n*-octyl) phthalate, which greatly simplifies the analysis. The possibility of contamination of biological samples with plasticizers in the laboratory is discussed briefly.

Keyphrases □ Bis(2-ethylhexyl) phthalate—GLC analysis in tissue and plasma □ Phthalate plasticizers, bis(2-ethylhexyl)—GLC analysis in tissues and plasma □ Plasticizers, bis(2-ethylhexyl) phthalate—GLC analysis in tissue and plasma □ GLC—analysis, bis(2-ethylhexyl) phthalate in tissue and plasma

Phthalic acid esters are widely used as plasticizers for polyvinyl chloride plastics. In particular, bis(2-ethylhexyl) phthalate (I) is contained in plastics utilized in the fabrication of medical devices such as blood storage bags, blood administration assemblies, hemodialysis units, and cardiopulmonary bypass units where it may represent 40% or more of the total weight of the finished plastic (1). Marcel and Noel (2) and Jaeger and Rubin (3) were the first to identify the presence of I in human blood stored in plastic bags. Since this discovery, considerable interest has developed regarding the potential toxicogenic effects of I and other phthalate esters, not only as the result of leaching from plastic medical devices but also from environmental contamination resulting from the many industrial and commercial uses of plastics (4).

During studies in this laboratory on the distribution and metabolism of I in experimental animals, it became apparent that its accurate quantitation in various organs by GLC was difficult with available techniques. Extraction of various tissue samples employing the method of Jaeger and Rubin (5) was not satisfactory. Attempted quantitation in which the GLC peak heights of accurately measured volumes of standard solutions of I were compared with those of solutions containing unknown amounts was unsatisfactory. Duplicate runs on the same tissue showed wide discrepancies due at least in part to the inherent difficulty in accurately measuring small samples for injection and to the fact that an average of 22 ± 8% was lost on extraction. These results suggested that a more reliable method of analysis might employ an internal standard. The validity of the use of an internal standard is discussed elsewhere (6), but it should be noted that once a suitable method has been developed, it is no longer necessary to be concerned with the complete recovery of extraction solvents nor with accurate measurement of the quantity injected. The internal standard chosen was bis(*n*-octyl) phthalate (II). The peak height ratio of I to II, when plotted against varying concentrations of I at constant concentration of II, was shown to be linear. Furthermore, it was shown that the peak height ratio of I to II was the same after addition to tissue and extraction in the manner described here as it was prior to tissue extraction. In other words, during the extraction process the relative amounts of both compounds lost were the same. Similar solubility characteristics of the internal standard and the substance analyzed, *i.e.*, II and I, respectively, are important criteria in the choice of an internal standard (7), and these results demonstrated that II is an excellent choice. Another case of an internal standard being employed in the GLC analysis of biological samples for I was reported (8), but it was applied only to blood and is unsuitable for tissue analysis due to interfering peaks. A recent paper described the determination of I in human plasma, but the method is more cumbersome and time consuming in that it involves column chromatographic separation of the plasticizer from the plasma, followed by GLC (9). The method is, however, more sensitive than the one described here.

EXPERIMENTAL

The following method is satisfactory for the analysis of I in many tissues, the extraction portion of the procedure being a modification of the method employed by Jaeger and Rubin (5). To 1–5 g of tissue (depending on whether the sample is likely to contain relatively large or small quantities of I) was added 1 ml of a chloroform solution containing 1.0, 0.25, or 0.1 mg of II[1] (the amount depending on the anticipated level of I). The tissue was then homogenized with 10 ml of methanol[2]–chloroform[2] (1:2), and the homogenizer was washed twice with 10-ml portions of the same solvent. The homogenate and washes were combined, shaken for 1 hr, placed in a separator containing an equal volume of 0.9% saline solution, and shaken. The lower layer (chloroform) was retained, the saline was washed once with 5 ml of methanol–chloroform (1:2), and this wash was combined with the chloroform layer. To the chloroform was added 2–3 g of silica gel[3] followed by shaking and suction filtration[4].

The sample was reduced to dryness under a stream of nitrogen, redissolved in 25 ml of methanol, centrifuged at 1000×*g* for 10 min, transferred to another tube, and reduced to dryness under a stream of nitrogen. Then the residue was dissolved in 1 ml of chloroform and analyzed by injection of 2–6 μl into a gas chromatograph[5] equipped with a flame-ionization detector employing a 1.82-m × 4-mm i.d. coiled glass column containing 3% OV-210 on 80–100-mesh Chromosorb WHP[6] under the following conditions: oven temperature, 225°; injector and detector temperature, 295°; and helium flow rate, 60 ml/min. A sensitivity of 1×10^{-9} was adequate for samples containing 1.0 or 0.25 mg of II; for samples containing only 0.1 mg of II, a sensitivity of 1×10^{-10} was required. The analysis was quite satisfactory for tissue containing a low lipid content such as liver, lung, and spleen. However, in the analysis of

[1] Eastman Organic Chemicals, Rochester, N.Y.
[2] Nanograde, Mallinckrodt Chemical Works, St. Louis, Mo.
[3] Analyzed reagent grade, J. T. Baker Co., Phillipsburg, N.J.
[4] Whatman No. 4 qualitative filter paper, U. & R. Balson Limited.
[5] Packard 7401, Packard Instrument Co., Downers Grove, Ill.
[6] Supelco, Inc., Bellefonte, Pa.

Table I—Comparison of Peak Height Ratio and Peak Height Methods in Spiked Lung Tissue

Actual Content of I, mg %	I:II	Peak Height
30	31	35
60	61	103
80	82	68
100	101	193
150	148	182
160	163	165

tissue containing large amounts of lipid, such as brain and fat, especially at the lower concentration of II, quantitation is difficult if not impossible due to the presence of substances possessing similar retention times to that of I and/or II.

For blood, plasma, or serum, extraction of 1 ml of the sample (after the addition of internal standard) with 30 ml of chloroform, followed by aspiration of the upper layer, evaporation to dryness, redissolution to 1 ml with chloroform, and subsequent injection was much faster than the tissue method with no loss of accuracy.

The I–II peak height ratio of the sample was compared to those obtained by graphing the peak height ratio of I–II standards *versus* concentration of I[1]. The standards employed contained varying amounts of I and equal quantities of II, the amount of the latter being the same as that added to the sample initially. Under these conditions the retention times of I and II were about 4 and 7 min, respectively, varying somewhat with changes in such parameters as column pressure and density of column packing.

RESULTS AND DISCUSSION

Table I illustrates the superiority of the peak height ratio method. Various samples of I were prepared in chloroform which also contained a constant amount of II (1 mg/ml of chloroform). The quantities of I chosen were within the range often found in lung tissue of animals injected intravenously with this compound. One milliliter of each of these samples was added to homogenized lung tissue and extracted, and the values were compared with those obtained by using only the peak height of I compared to the peak height of standards. The precision of the analysis for I in a concentration of 1–10 mg % was approximately ±20%, whereas duplicate samples were usually reproducible to within ±10% in concentrations above this level.

In another experiment, a mongrel dog (11 kg) was infused intravenously over about 30 min with I, prepared by sonication in dog plasma, at a dose of 25 mg/kg. Six hours later the dog was sacrificed with sodium pentobarbital[7], and the lung and liver were removed and minced. These tissues were then divided into seven approximately equal samples, stored in a refrigerator freezer at −11.5°, and analyzed weekly. The results (Table II) demonstrate that the ratio method yields quite reproducible results and indicate that there is little appreciable deterioration of I in the samples when stored under these conditions since the difference between the first and last assays for both tissues is within the experimental error given. The liver samples were determined with II at the 0.25-mg/ml level, whereas the lung samples were analyzed with the internal standard at a concentration of 1.0-mg/ml.

The minimum detectable level of I in lung, liver, and spleen tissues as well as plasma is about 1 mg %. However, the difficulties encountered in the analysis of samples containing less than 5 mg % should be emphasized. At a sensitivity of 1×10^{-10}, with II present at the 0.1-mg/ml level, standards containing I in the range of approximately 1–10 mg % were suitable. At these low levels of I and high sensitivity, it is essential that adequate measures be taken to assure the least possible contamination from glassware, solvents, *etc.*, and that careful blank determinations be performed. It has been found in this laboratory that small amounts of phthalates are present in silicic acid as well as in some solvents and filter paper. Other investigators (10) made similar observations. The described analytical procedure avoids the use of phase-separating filter paper which has been found to contain phthalates (10). Even when all of these precautions were taken, blanks still contained approximately 0.2 mg %. All traces of phthalate, however, can be removed by soaking glassware overnight in concentrated sulfuric acid. When attempting to detect I at these levels, it is also important to be careful in the storage and handling of the sample so that it is protected from contamination. For example, samples should not be stored in screw-top glass containers if the cap is lined with polyvinyl chloride or metal foil. All-glass containers are preferable.

[7] Pentathesia, Wittney and Co., Denver, Colo.

Table II—Amount (mg %) of I Present in Dog Lung and Liver Tissue

Sample	Liver	Lung
1	11.6	143.0
2	10.9	129.3
3	10.2	135.2
4	7.6	133.1
5	9.3	127.4
6	9.7	137.9
7	9.8	129.3
Mean	9.9	133.6
SD	1.3	5.6

There are many reports of the occurrence of small quantities of phthalate esters in numerous samples of biological origin. In light of the facts presented, the possibility exists that, at least in some of these cases, the contamination occurred in the laboratory and not in the environment.

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