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Measurement of Phthalates in Small Samples of Mammalian Tissue

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Di-(2-ethylhexyl)-phthalate (DEHP) is a phthalic acid ester that is used as a plasticizer in polyvinyl chloride products, many of which have widespread medical application. DEHP has been shown to be leached from products used for storage and delivery of blood transfusions during procedures such as plasmaphoresis, hemodialysis and open heart surgery (Thomas and Thomas 1984). Results of studies in this laboratory (Crocker et al 1983) have suggested that there is an association between the absorption and deposition of DEHP (and/or related chemicals) in the kidney and the acquired renal cystic disease (ACD) frequently seen in patients who have undergone prolonged dialysis treatment (Dunnill et al 1977). In order to determine the relationship between the two, it has been necessary to establish a method for extracting and accurately quantitating minute amounts of these chemicals in small tissue samples. We have now established such a method using kidneys from normal rats and from a rat model for ACD.

MATERIALS AND METHODS

Acetonitrile (ACS grade), petroleum ether (PE) (pesticide grade), diethyl ether (DEE), iso-octane (HPLC grade), sodium chloride and anhydrous granular sodium sulfate were obtained from Fisher Scientific (Canada). Florisil (60–100 U.S. mesh) was obtained from BDH Chemicals (Toronto, Canada). DEHP was purchased from ICN Pharmaceuticals (Plainsview, NY) and N-dibutyl-phthalate (NBP) was from Sulpelco Canada Ltd. (Oakville, Canada). [UL-^{14}C]-Di(2-ethylhexyl)-phthalate (7.32 mCi/mmol) was purchased from Pathfinder Laboratories Inc. (St. Louis, MO). All water was doubly-distilled and charcoal filtered to remove any contaminating organic material.

Sprague Dawley rats (175–200 g) (Charles River Canada Inc., St. Constant, Canada) were housed in the Animal Quarters of Dalhousie University for twelve months prior to analysis under the guidelines of the Canadian Council on Animal Care. Animals were kept in metal hanging cages and glass water bottles were used. Rats were killed by intraperitoneal injection of phenobarbital

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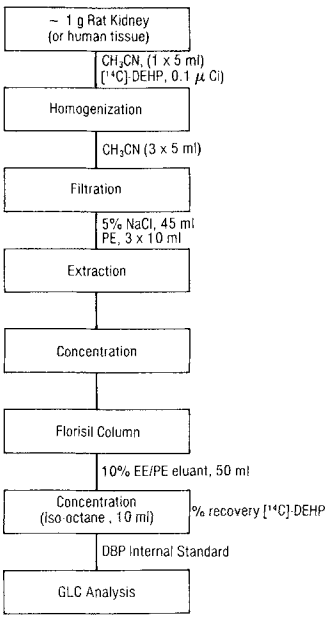

Figure 1. Extraction and analysis of DEHP from rat tissue.

(Euthanol) (MTC Pharmaceutical, Hamilton, Canada). Abdomens were opened at the midline and each kidney was removed from the retro peritoneal fascia with stainless steel forceps. The renal pedicle was bisected with scissors, and the kidneys were divided longitudinally through the cortex and pelvis. The tissue was then placed in aluminum foil and stored at -70°C until it was extracted and analyzed for DEHP content.

The procedure for DEHP extraction summarized in Fig. 1 is based upon the method of Giam et al (1975). Approximately 1 g of kidney tissue was transferred to a glass mortar together with 5 g Na_2SO_4 and 5 ml acetonitrile. For the recovery studies, approximately 0.1 uCi (200,000 dpm) of [^{14}C] DEHP was added, and the mixture was homogenized with a glass pestle for 5 minutes. The homogenate was filtered into a 125 ml separatory funnel through a glass funnel that had been plugged with glass wool and anhydrous Na_2SO_4. The residue was rinsed three times with 5 ml acetonitrile. Forty-five ml of a saline solution (5% NaCl, w/v) was added to the filtrate and this mixture was extracted three times with 10 ml petroleum ether. The organic phases were collected and pooled, and were reduced to 5 ml under a gentle stream of nitrogen (N_2). The extract was cleaned by filtration through Na_2SO_4. It was then placed on a glass column (1x20 cm) containing 7 g of florisil that had been activated at 300°C for four hours and subsequently deactivated by agitation with water (3%, w/v) for ten minutes. Fifty ml of a solution of diethyl ether/petroleum ether (10/90, v/v) was used to elute the DEHP fraction. In those experiments in which recovery of [^{14}C] DEHP was being monitored, aliquots of eluant

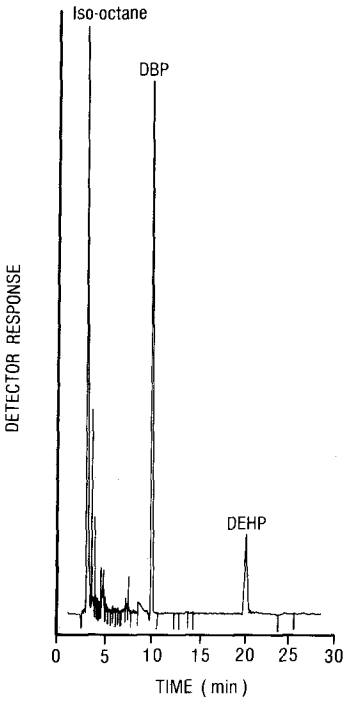

Figure 2. GLC analysis of DEHP from rat kidney.

were counted at several points during the extraction procedure. In studies in which DEHP was being quantitated, the column eluant was concentrated under N_2 and resuspended in a small volume of iso-octane. A known amount of N-dibutyl phthalate (DBP) was added to each sample as an internal standard and the components were separated by GLC (Fig. 2).

A Sigma 3B gas-liquid chromatograph (Perkin-Elmer (Canada) Ltd., Montreal, Canada) equipped with a 30 m SP-2330 capillary column (Supelco Canada Ltd.) and flame ionization detector was used to analyze tissue extracts. The injector, detector, and column temperatures were 230°C, 230°C, and 215°C, respectively. Helium (60 ml/min) was used as the carrier gas. An LCI-100 Laboratory Computing Integrator (Perkin-Elmer (Canada) Ltd.) was used to quantitate DEHP by comparison with a known amount of the internal standard, DBP. Retention times for DBP and DEHP were 10 and 20 min, respectively (Fig. 2).

The recovery of DEHP was determined by measuring the amount of radioactivity before and during column separation. Duplicate aliquots from each fraction were taken to dryness and radioactivity was measured on a Beckman 5801 Liquid Scintillation Counter.

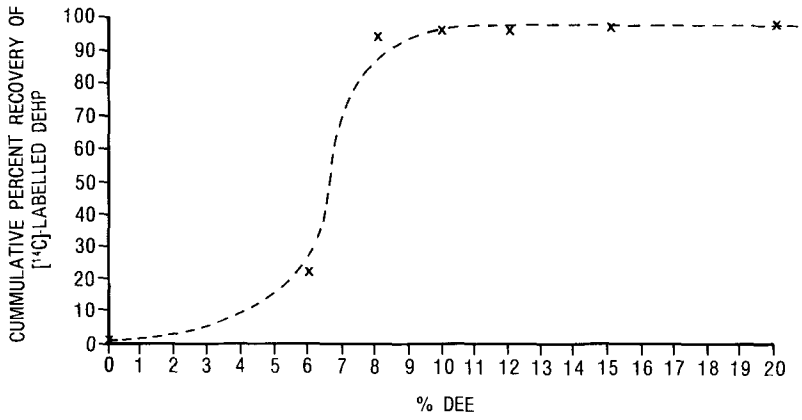

Figure 3. Recovery of [^{14}C]DEHP from the florisil column with increasing amounts of DEE. The columns were eluted with 50 ml volumes of DEE/PE that contained the percent DEE/PE (v/v) indicated in the abcissa.

RESULTS AND DISCUSSION

The extraction method proved to be very efficient with a pre-column recovery of 93.4 percent and the overall recovery of DEHP of greater than 87.5 percent.

The relative proportions of DEE and PE in the eluant had an appreciable influence on the efficiency of extraction of [^{14}C] DEHP from the florisil column (Fig. 3). Maximum recovery was achieved when the proportions of DEE/PE were 10/90 (v/v). Recovery was not improved with further increases in the proportion of DEE.

In order to determine the sensitivity and linearity of the analysis, samples of rat kidney were spiked with known amounts of non-radioactive DEHP at the initial homogenization step. As shown in Figure 4, this assay provides a sensitive method for measuring small quantities of DEHP in as little as 1 g of tissue. This gives it a distinct advantage over the method described by Giam et al (1975) which requires 30-100 gms of tissue. Also, we have used a combination of flame ionization detector (FID) and fused silica capillary column with very reproducible results. The electron capture detector used by Giam et al (1975) may be more sensitive than FID, but runs a greater risk of 'noise' due to background contamination of DEHP. With our technique, almost 99% of the DEHP is extracted from the florisil column with 10% DEE/90% PE, while Giam et al (1975) did not observe quantitative recovery until the proportion of DEE was increased to 15%. The reason for this difference is not known, however it may relate to minor differences in the preparation of the florisil, since it is known that small changes in the activation/deactivation process can dramatically affect elution patterns. Chen et al (1979) have also developed an assay for small amounts of tissue, but their technique has a

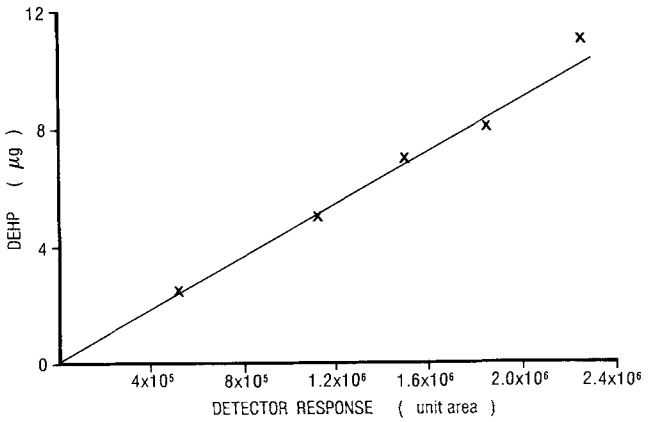

Figure 4. Relationship between the amount of DEHP added to kidney homogenates and the detector response during GLC

slightly lower recovery (circa 80%).

When assay procedure was used to measure the DEHP content of renal tissue of healthy rats that had no known contact with plastic products, phthalate concentrations were 13.7 ± 7.9 ug/gram of kidney tissue (mean ± SD: n = 7). Concentrations of DEHP in renal tissue from animals that had been given phthalates depended on the degree of renal function. These studies have been reported elsewhere (Crocker et al 1986).

There is an increasing concern regarding possible associations between environmental chemicals and disease processes. A relationship between tissue phthlate levels and ACD may exist, since the latter afflicts up to 40% of long-term hemodialysis patients (Dunnill et al 1977). Furthermore, these patients demonstrate significant potential for malignant transformation (Hughson 1985). Patients in chronic renal failure have an increased contact with DEHP not only from frequent dialysis procedures but also from frequent blood transfusions and i.v. fluids (Gibson et al 1976). The assay described in this report provides a useful tool with which to address this question in both animal models and in human diseased tissue. Future adaptations of this technique to other organic materials will be of great value to researchers in toxicology.

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