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DETERMINATION OF DI-(2-ETHYLHEXYL)PHTHALATE IN HUMAN PLACENTA

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SUMMARY

A simple method has been devised for the analysis of di-(2-ethylhexyl)phthalate (DEHP) in human placenta which relies entirely on three rapid partition steps prior to gas chromatography with electron capture detection. The use of adsorption chromatography with all its attendant disadvantages for sample clean-up is eliminated in this procedure. Placental samples taken from women who had given birth to a normal baby contained 0.06 $\pm$ 0.02 ppm of DEHP. The principal limitation of this method, and of any others, is the high blanks given by laboratory equipment and solvents. The reduction of contamination to workable levels is described.

INTRODUCTION

A potential environmental monitor for man, and because of its unique relationship to the fetus, a monitor of fetal exposure, is the placenta. The human placenta is a relatively large organ (300–600 g), readily available after the birth and of a well defined age. It offers the possibility of studying the effect of nine months environmental insult on a new tissue as well as providing us with our best chance of relating fetal exposure to fetal damage. Genetic variance is obviously an important factor in fetal malformations but environmental causative agents are also being cited as playing a role in this area. A few scattered reports on the levels of trace metals[1–10], selenium[10,11], iodine[12] and organochlorine insecticides[13–15] in human placentae have been recorded.

There is growing concern for the effects of trace levels of di-(2-ethylhexyl)-phthalate (DEHP) in the environment, a substance widely used in industry as a plasticizer. It is estimated that the annual production of this chemical in the mid-1970's will amount to approximately 1.5×10^9 lbs. in the U.S.A. alone[16]. Recent studies have shown that at trace levels it impairs the reproductive efficiency of fish as well as acting as a heart rate depressant[17–19]. At relatively high levels, it has been demonstrated to be a teratogen in mice[20], rats[21,22] and chicks[23,24]. Concern has also been expressed about the leaching of comparatively large quantities of plasticizer into blood stored in plastic bags[25–27], from the use of plastic tubing etc. in life-maintaining machines[25,28], and also from plastic devices used during surgical operations[29,30]. The

environmental chemistry of phthalate esters has been the subject of several recent reviews[16,31,32].

Apart from the references cites above, very little information on the levels of DEHP in man is available. This is the first report of the determination of DEHP in human placenta.

EXPERIMENTAL

DEHP and dinonyl phthalate (DNP) were obtained from Phase Separations (Queensferry, Great Britain). DNP was used as an internal standard (10 μl) of a solution containing 2 mg/ml in acetonitrile.

Water was distilled in an all-glass apparatus from acid permanganate, then extracted consecutively with *n*-hexane until free of phthalate esters as determined by gas–liquid chromatography with electron capture detection (GLC–ECD) of the hexane phase.

n-Hexane obtained from BDH (Poole, Great Britain) was purified by distillation from solid potassium hydroxide in an all-glass apparatus through a Fenske ring column (1.2 m × 3.0 cm).

Acetonitrile obtained from Fisons (Loughborough, Great Britain) was distilled in an all-glass apparatus through a Fenske ring column (as above).

Dimethylformamide–water (94:6) was extracted consecutively with *n*-hexane until free of phthalate esters as determined by GLC–ECD analysis of the hexane phase.

Glass fibre filter papers (Whatman GF/A; Whatman, Maidstone, Great Britain) were decontaminated by heating in an oven at 250 °C for at least 12 h.

All glass apparatus was washed in detergent, thoroughly rinsed with water and then soaked in chromic acid overnight. The apparatus was then rinsed with water and baked in an oven at 250 °C for at least 12 h.

Silicone rubber GLC septa, aluminium foil and stainless-steel apparatus were heated in an oven at 250 °C for at least 12 h. When not used immediately, and while cooling, all apparatus was covered with aluminium foil. Placental samples were homogenised in glass beakers using a stainless-steel ultra-turrax tissue grinder.

GLC was carried out with a Pye Unicam GCV using either flame ionization detectors (FID) or a constant-current electron capture detector. The latter was operated with a detector current of 10 nA, a sensitivity × 64 and a detector oven temperature of 260 °C. Extracts were analysed on a 0.9 m × 6.25 mm O.D. column of 3% OV-101 on Supasorb (AW HMDS, 100–120 mesh) with a column temperature of 240 °C and a nitrogen flow-rate of 30 ml/min (retention times (t_R): DEHP = 4.9 min, DNP = 6.7 min). Second column confirmation was carried out with a 1.5 m × 6.25 mm O.D. column of 3% OV-17 on Supasorb (AW HMDS, 100–120 mesh) with a column temperature of 250 °C and a nitrogen flow-rate of 35 ml/min (t_R: DEHP = 6.3 min, DNP = 8.3 min).

Placentae were obtained from the hospital wrapped in aluminium foil. On arrival, they were allowed to drain to remove excess blood and then rinsed superficially with water. If not analysed immediately, they were stored in a deep freeze at −30 °C. Approximately 80 g of tissue were cut into small pieces, the internal standard added and the sample homogenized in acetonitrile (1 ml/g). The homogenate was

filtered under vacuum through a glass sinter funnel (porosity 3) supporting a glass fiber filter paper, directly into a separating funnel (500 ml) with PTFE stopcock. The solid residue and glass fiber filter paper were homogenized once more with a second volume of acetonitrile and filtered as before. To the acetonitrile was added water (equal to half the volume of acetonitrile used) and the aqueous phase extracted twice with hexane (40 ml). The hexane was evaporated *in vacuo* and the residue partitioned between dimethylformamide–water (94:6) and hexane (5 ml of each phase). The dimethylformamide phase was extracted with a further volume of hexane (5 ml) and the hexane layers combined and evaporated to a residue *in vacuo*. This was taken up in hexane (5 ml) and extracted three times with acetonitrile saturated with hexane (3 × 5 ml). The acetonitrile phase was evaporated to a residue, re-dissolved in acetonitrile (3 ml) and 2–5 μl used for analysis by GLC–ECD. The analytical procedure is summarized in Fig. 1.

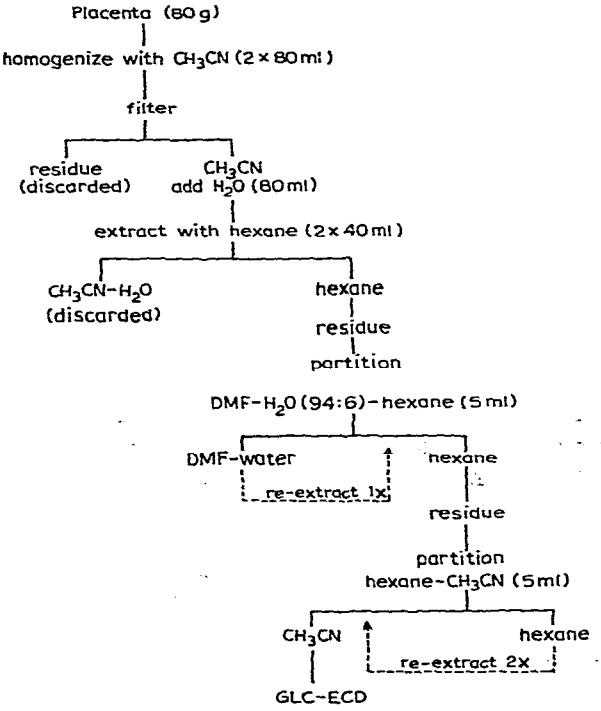

Fig. 1. The analytical scheme for the determination of DEHP in human placenta.

RESULTS AND DISCUSSION

Plastics find many uses in everyday life, and the laboratory is no exception. As a consequence, the principal problem in analysing trace levels of plasticizers is the reduction of background levels from solvents and equipment to such an extent that they become negligible by comparison to the analytical signal. All normal laboratory apparatus and chemicals were found to contain significant amounts of DEHP and had to be treated prior to use. Contact of the sample was limited to glass, aluminium

foil, stainless-steel or PTFE as these represented the materials which could be most readily decontaminated. Cleaning procedures are given in the experimental section and were strictly adhered to. The use of "muffling" for the decontamination of laboratory equipment is both efficient and simple[33–35].

The level of contamination of DEHP in solvents varied both with the commercial source and within the batch from any one source. Solvents showing little contamination were further purified by all-glass distillation through a Fenske ring column. Attempts at purifying solvents with a moderate level of contamination by the method of Williams[36] or by treatment with concentrated acids, aqueous alkaline potassium permanganate or lithium aluminium hydride met only with partial success. Although a moderate reduction in the level of DEHP was achieved, this was offset by the appearance of additional peaks in the chromatogram and a general broadening of the solvent front. With solvents containing low levels of DEHP, significant contributions to the blank value were obtained from syringes and GLC septa. Standard GLC syringes of the removable needle type containing a PTFE seal had a pronounced memory effect. After the injection of a concentrated sample for FID analysis, it was often very difficult to clean this type of syringe by the solvent flush technique for use with the ECD. Syringes with a stainless-steel needle welded to the glass body were easier to clean in this respect. GLC septa were found to be an additional source of DEHP on the chromatogram as well as having a memory effect after the injection of concentrated samples[27]. The use of PTFE faced septa offered no advantages over the normal silicone rubber variety. Heating the septa for 12 h at 250 °C in an oven and operation of the gas chromatograph with the injection port heater set to 250 °C gave acceptable results. To avoid cross-contamination, different septa and syringes were used for FID and ECD analysis. By the use of these techniques, the final background level of DEHP was reduced to zero at the sensitivity setting used on the ECD for placental sample analysis.

Acetonitrile was found to be the best solvent for extracting DEHP from placenta. Acetone and methanol were equally as effective, but acetone gave complex chromatograms due to extraction of unwanted material and methanol tended to form emulsions readily on addition of water. Samples fortified with DEHP showed a virtually quantitative recovery by a double homogenization and filtration of placental samples with acetonitrile. The residues from undoped placentae after treatment as above and then exhaustively Soxhlet extracted with the same solvent overnight yielded insignificant amounts of DEHP.

The hexane phase from the water–acetonitrile extraction when analysed by GLC–ECD contained too many electron capturing co-extractants for quantitative work. The use of florasil, alumina or silica gel column chromatography and silica gel thin-layer chromatography (TLC) have been described for the clean-up of biological samples containing DEHP prior to GLC[16,34,35]. We found that all column methods are slow and that DEHP with ether–hexane mixtures elutes as a broad tailing peak requiring a large volume of solvent for its complete removal. Column adsorbents were invariably contaminated with plasticizers and required careful heat treatment and then deactivation before use. The use of TLC adds to the contamination problem, not only from the adsorbent but also from the glass backing plates, the spreading equipment and by atmospheric contamination while drying. A triple elution in acetonitrile followed by re-activation was necessary to give acceptable blank values.

However, removal of DEHP from the adsorbent by elution from packed micro-columns suffered from the same problems as column chromatography and direct methods such as disruption of the silica gel with methanol in an ultrasonic bath or addition of water to the silica gel in a small vial followed by extraction with hexane gave very poor recoveries (usually $< 50\%$). In the light of these difficulties it was decided to investigate the further use of partition systems.

The approximate partition coefficients for DEHP with some immiscible solvent combinations are given in Table I. It was found that partition between hexane and dimethylformamide (DMF), dimethyl sulphoxide or propylene carbonate were particularly effective in removing electron capturing coextractants. Of these solvent combinations, dimethyl formamide was the most useful, but the partition function was too small to give high recoveries of DEHP without recourse to countercurrent techniques. The addition of a small amount of water to DMF favours the partition of DEHP into hexane without excessive extraction of interfering substances. Direct injection of the hexane phase gave a clear indication of DEHP but often showed excessive tailing of the solvent front into the analytical region of the chromatogram. Extraction of the hexane phase consecutively with acetonitrile ($3 \times$) followed by injection from the acetonitrile phase gave a further reduction in the size of the solvent front. A typical chromatogram from a placental extract is shown in Fig. 2. The proposed analytical scheme (Fig. 1) allows a total recovery of approximately 80% of the DEHP present in the sample. The principal loss occurring at the last partition stage between acetonitrile and hexane. The use of DNP as internal standard (no DNP has been found in any placental samples analysed in this laboratory) adequately corrects for this and for any other experimental losses.

TABLE I

APPROXIMATE PARTITION COEFFICIENTS FOR DEHP

Immiscible solvent pair	*Partition coefficient*
Cyclohexane–acetonitrile*	3.0
Hexane–methanol**	1.2
Hexane–acetonitrile	1.6
Hexane–acetonitrile–water (2:1:1)	205
Hexane–dimethylformamide	1.2
Hexane–dimethylformamide–water (100:94:6)	25

* A centrifuge is required to separate phases.
** Methanol must be saturated with hexane.

Several GLC phases were evaluated for the analysis of phthalate esters. The silicone oil phases were found to be particularly suitable and gave good peak shape without evidence of peak tailing. It was noticed, that new columns which had settled on conditioning, gave tailing peaks unless sufficient additional packing was added so that on injection the syringe needle reached the surface of the column material. Polyester and carbowax phases were excellent for the separation of the volatile phthalate esters but had long retention times and gave poor peak shapes wih DEHP and DNP. The high temperatures required for the analysis of DEHP on these columns

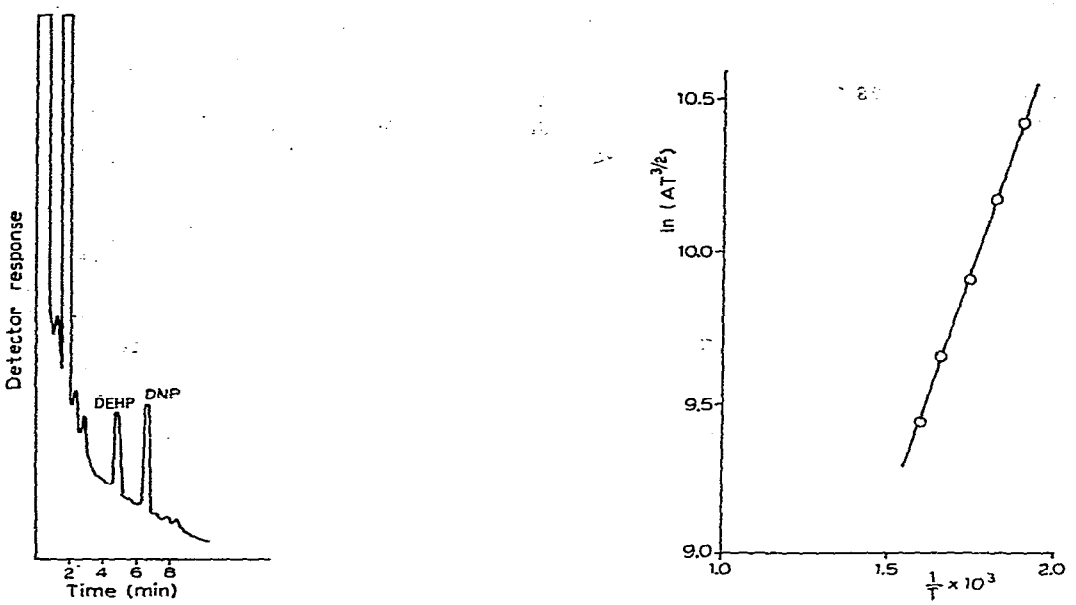

Fig. 2. GLC of the acetonitrile extract of placenta (see Experimental for details). After the elution of DNP, 10 min were allowed to elapse between injections to remove less volatile material from the column.

Fig. 3. The effect of detector oven temperature on the response of the ECD towards DEHP. A = peak area of a fixed mass of DEHP; T = detector oven temperature (°K).

was incompatible with the operation of the ECD at high sensitivity due to column bleed.

Phthalate esters are good electron capturing compounds and can be determined both selectively and sensitively with an electron capture detector[37,38]. The molar reponses of the detector is highest for the lower molecular weight homologues and decreases with increase in molecular weight[39]. The detector response to the phthalate esters shows a marked dependence on the carrier gas flow-rate and the detector oven temperature. The effect of detector oven temperature on detector response is shown in Fig. 3, for a plot of ln $AT^{3/2}$ *vs.* $1/T$ (A is area of the peak for a fixed mass of phthalate ester and T detector oven temperature, °K)[40,41]. The slope of the line indicates a non-dissociative electron capture mechanism. The highest detector response is observed with low detector oven temperatures. The detector response also increases as the carrier gas flow-rate is reduced. The selected flow-rate is of course a compromise between the most favourable conditions for the detector response and for the chromatography. Details of the optimum conditions are given in the experimental section.

To determine background environmental levels of DEHP, ten placenta were selected from women in the Birmingham area who had given birth to a normal baby. The levels of DEHP found were 0.06 ± 0.02 ppm on a fresh weight basis (placenta contains approximately 85% water [w/w]). By comparison with the levels of DEHP reported as present in human blood (20 ppm)[42], the level found in placenta might have been expected to be higher. The levels of DEHP perhaps reflect the low lipid

content of this tissue (approximately 0.4%, w/w)[43]. It has been shown that in blood, the DEHP is almost exclusively found in the plasma lipoprotein fractions[27].

CONCLUSIONS

A rapid method for the determination of DEHP by GLC–ECD which can be applied to human placental samples has been described. Sources of contamination, which constitutes the principal limitation on sensitivity, have been identified and methods for their reduction given.

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