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Inhibition of 15-Lipoxygenase by Phthalate Plasticizers

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Alkyl esters of phthalic acid, such as di-(2-ethylhexyl) phthalate, are commonly used as plasticizers in polyvinyl chloride and other plastics. They may constitute 20-40% of the weight of the plastic; production per year is 3-4 million tons; and it has been estimated that in the Netherlands alone, 100,000 tons of di-(2-ethylhexyl) phthalate is present in landfills (Warns 1987). Phthalates may leach from plastic containers and wrappers into transfusion fluids, food and bottled drinking water (Plonait et al. 1993; Page and Lacroix 1995; Fayad et al. 1997). The normal daily intake has been estimated at 0.5-2 mg/day, while doses of up to 300 mg may be received during exchange transfusion (Warns 1987).

Numerous biological effects of phthalate esters have been reported (Review: Rettenmeier and Mettang 1997). The influence of phthalates on mammalian fertility and reproduction has been intensively researched. Phthalates induce testicular damage (Fukuoka et al. 1994; Parmar et al. 1995), decrease sperm density (Siddiqui and Srivastava 1992; Murature et al. 1987) and motility (Fredricsson et al. 1993), and interfere with sperm metabolic enzymes (Siddiqui and Srivastava 1992). It has been suggested that increased exposure to phthalates may be partially responsible for the decreasing values of sperm density reported in recent decades (Murature et al. 1987). Thus, phthalates cannot be regarded as innocuous compounds in spite of their low acute toxicity, and exposure to phthalates has repeatedly been regarded as a source for concern (Warns 1987; Morgenroth 1993; Malik et al. 1994). Recently, the finding of phthalates in milk powder for babies has caused considerable public attention (Anonymous 1996). Increased knowledge of the biological activity of phthalate esters would thus seem to be of importance.

Somewhat serendipitously, we observed that 15-lipoxygenase (15-LO) inhibition by a plant extract was in part due to the presence of di-(2-ethylhexyl) phthalate as a contaminant. Since modulation of lipoxygenase activity by phthalate esters does not appear to be well known, we decided to study this effect further. The results are reported in this communication.

MATERIALS AND METHODS

Di-*n*-butyl phthalate, di-(2-ethyihexyl) phthalate and quercetin dihydrate were obtained from Koch-Light (Haverhill, UK). Di-*n*-octyl phthalate was from Eastman (Rochester, NY, USA); soybean lipoxygenase type 1-B and linoleic acid from Sigma (St.Louis, MO, USA) and phthalic acid from Merck (Darmstadt, Germany).

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Other chemicals were of the highest purity available. The identity and purity of the phthalates were checked by ^{1}H NMR spectroscopy. All measurements were carried out in a Shimadzu UV-160A spectrophotometer equipped with a CPS-240A thermostatted cell positioner (Shimadzu, Kyoto, Japan) at a temperature of 21°.

Lipoxygenase activity was measured as described previously (Lyckander and Malterud 1992) in borate buffer (0.2*M*, pH 9.00) by the increase in absorbance at 234 nm from 30 to 90 sec after addition of the enzyme, using linoleic acid (134 μ*M*) as substrate. The final enzyme concentration was 167 U/ml. Test substances were added in DMSO (final DMSO concentration 1.6%); DMSO alone was added in uninhibited control experiments. All measurements were carried out at least twice, in each instance using six or more parallels of controls and three or more parallels for each concentration of the test substances.

Calculation of enzyme activity was carried out as previously described (Lyckander and Malterud 1992), and IC_{50} values were determined by linear interpolation between the measuring points closest to 50% activity. Values are expressed as means ± SD. Student's t-test was employed for determination of statistical significance.

RESULTS AND DISCUSSION

The three phthalates tested all inhibited soybean 15-lipoxygenase, an enzyme which peroxidizes polyunsaturated fatty acids (see Table 1). To our knowledge, this is a biological activity hitherto unreported for these compounds, although a general decrease in levels of cyclooxygenase and lipoxygenase products in rat peritoneal leukocytes exposed to phthalate esters has been reported (Tavares and Vine 1984). Although di-(2-ethylhexyl) phthalate had the lowest IC_{50} value, it appears that the alkyl chain of the ester is of minor importance, since di-*n*-octyl phthalate and di-*n*-butyl phthalate had IC_{50} values comparable to di-(2-ethylhexyl) phthalate. A slight tendency towards higher inhibitory activity with larger alkyl moieties may, however, be present. The ester groups appears essential for the inhibitory activity, phthalic acid itself being considerably less active.

Table 1. Inhibition of soybean 15-lipoxygenase by phthalates, phthalic acid and quercetin (positive control). IC_{50}: Concentration for 50 % inhibition of the enzyme.

Substance	IC_{50} (μ*M*)
Di-(2-ethylhexyl) phthalate	93 ± 2
Di-*n*-octyl phthalate	99 ± 5
Di-*n*-butyl phthalate	108 ± 7
Phthalic acid	218 ± 11
Quercetin (positive control)	68 ± 5

As a positive control, quercetin (3,5,7,3',4'-pentahydroxyflavone) was employed, an effective lipoxygenase inhibitor which has been extensively studied and which we have used previously for this purpose (Lyckander and Malterud 1992). The

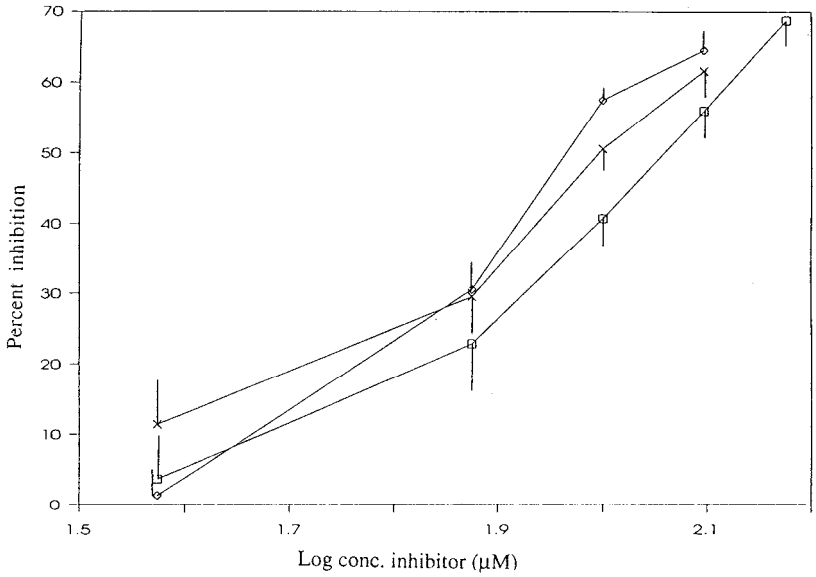

Figure 1. Inhibition of soybean 15-lipoxygenase by di-*n*-butyl phthalate (□), di-*n*-octyl phthalate (×) and di-(2-ethylhexyl) phthalate (◇).. Vertical bars denote SD. Inhibition is significant ($P < 0.01$) at all concentrations except the lowest one.

difference between our present IC_{50} value for quercetin and the one previously reported by us, 98±3 μ*M*, may be due to different batches of the enzyme. Concentration - activity curves for the phthalates are shown in Fig. 1. At high concentrations (above 125 μ*M* for di-(2-ethylhexyl) phthalate and di-*n*-octyl phthalate, 150 μ*M* for di-*n*-butyl phthalate), poor reproducibility was obtained. This was probably due to formation of microdroplets (which was observed as clouding of the assay solution), since phthalate esters are hydrophobic substances with limited solubility in water. Di-*n*-butyl phthalate, which has the shortest alkyl chain and presumably is less hydrophobic, appeared to be somewhat better soluble than the two other substances.

Inhibition of soybean 15-LO is predictive for inhibition of mammalian 15-LO (Nuhn et al. 1991; Gleason et al. 1995). 15-Lipoxygenase is present in prostasomes (Oliw et al. 1993) and is important for the acrosome reaction in mammalian spermatozoa (Lax et al. 1990; Breitbart et al. 1995), a reaction which is essential to fertilization, being required for the penetration of the zona pellucida by sperm cells and for their fusion with the oocyte plasma membrane. The importance of arachidonate metabolites for the acrosome reaction has, however, been debated (Mack et al. 1992).

We conclude that phthalates are inhibitors of 15-lipoxygenase, an effect that has not been reported previously. Although their activity is moderate and their concentration in testis and spermatocytes at present appears unknown (serum concentrations of up to 21.6 μg/ml; ca. 5.5 μM have, however, been reported (Plonait et al. 1993)), further investigation of their possible deleterious role towards human fertility by hindering the acrosome reaction through lipoxygenase inhibition seems worth while.

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