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## Note

# Determination of di- and mono(2-ethylhexyl) phthalate in plasma by gas chromatography

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Di(2-ethylhexyl) phthalate (DEHP) is used as a plasticizer in the manufacture of PVC plastics, sometimes up to a concentration of 30—40%. Many studies have shown that DEHP can be leached from the PVC matrix and contaminate the environment. For example, it can accumulate in blood stored in plastic blood bag assemblies [1]. There is therefore major concern about the possible health hazards associated with the use of DEHP, especially as it has been shown to induce liver tumours and testicular atrophy in rats and mice [2—4]. Mono(2-ethylhexyl) phthalate (MEHP), which is formed by hydrolysis of one ester linkage, is a major metabolite of DEHP [5] and is suspected to play an important role in the induction of the toxic effects observed after administration of DEHP [6].

Several methods have been proposed for the determination of DEHP and MEHP in plasma, including gas chromatography (GC) with flame ionization [7, 8] and electron-capture detection [9—11] and high-performance liquid chromatography [12]. The time-consuming sample preparation [9], the use of temperature programming [7], the absence of an internal standard [7, 12] or the use of only one internal standard for both DEHP and MEHP [8, 9], and the use of large amounts of solvents [7] make these methods unsuitable for routine purposes. Moreover, the alkylation of MEHP was performed previously by treating the extract with diazomethane [7, 8, 10], a hazardous compound, or by a time-consuming solid—liquid phase-transfer catalysis, giving rise to the hexyl ester [9]. Finally, the use of large plasma samples precludes the application of these methods to the study of the toxicokinetics of DEHP in rats.

This paper presents a rapid, simple and sensitive GC method, using flame ionization detection, for the determination of DEHP and MEHP in small

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plasma samples. Preliminary data are given on the plasma concentrations of DEHP and MEHP after oral administration of DEHP in rats.

## EXPERIMENTAL

### *Chemicals and reagents*

DEHP (Essochem Europe, Machelen, Belgium) was used as received and MEHP (British Petroleum, Sully, U.K.) was purified before use [13]. Mono- and di-*n*-octyl phthalate were synthesized according to the method of Albro et al. [13]. The purities (> 99%) of all compounds were checked by GC and thin-layer chromatography. *n*-Hexane (UCB, Brussels, Belgium) was of pesticide grade. Silicic acid (Sigma, St. Louis, MO, U.S.A.) was rinsed with *n*-hexane before use. The derivatization reagent, tetrabutylammonium hydroxide (Chrompack, Merksem, Belgium), was used as a 0.05 *M* solution in methanol—chloroform (1:3). All other reagents and solvents used were of analytical-reagent grade.

### *Glassware*

All glassware was silanized by soaking for 1 h in a 5% solution of trichloromethylsilane in xylene, rinsed with xylene and methanol, dried for 1 h at 100°C and rinsed with *n*-hexane before use.

### *Gas chromatography*

A Varian 2100 gas chromatograph, equipped with a dual flame ionization detector, was used. GC was performed on 1.8 m × 2 mm I.D. glass columns packed with Gas-Chrom Q coated with SE-30 (Alltech Europe, Eke, Belgium), either at 2% for DEHP or at 5% for MEHP, with a nitrogen flow-rate of 30 ml/min. The temperatures for both compounds were: column 190°C, injection block 270°C and detector 250°C. The hydrogen and air flow-rates were 60 and 300 ml/min, respectively. The peak areas were recorded on a Hewlett-Packard 3380A recording integrator. Both columns were kept in the gas chromatograph throughout.

### *Extraction and derivatization*

A 300-μl volume of acetonitrile containing 10 μg of both internal standards (di-*n*-octyl phthalate for DEHP, and mono-*n*-octyl phthalate for MEHP) and 200 μl of water were added to 100 μl of plasma in a 10-ml glass-stoppered centrifuge tube.

For the determination of DEHP, the mixture was extracted with 3.0 ml of a 1.5 g% suspension of silicic acid in *n*-hexane by shaking horizontally for 5 min. After centrifugation for 5 min at 3000 *g*, the organic layer was transferred with a Pasteur pipette into a 6-ml glass-stoppered conical tube, and the organic layer was removed under a gentle stream of nitrogen at room temperature. The walls were washed with 300 μl of *n*-hexane and the solvent was evaporated under nitrogen. For GC analysis, the residue was dissolved in 10 μl of chloroform and an aliquot was injected into the gas chromatograph.

For the determination of MEHP, 2 ml of 0.1 *M* citrate buffer (pH 2) were added to the remaining aqueous phase, which was then extracted twice with

3.0 ml of $n$-hexane. The sample was processed further as for DEHP. For GC analysis the residue was dissolved in 10 μl of a 0.05 $M$ tetrabutylammonium hydroxide solution in methanol—chloroform (1:3) and 1 μl was injected into the gas chromatograph.

*Calibration*

Di- and mono-$n$-octyl phthalate were selected as internal standards for DEHP and MEHP, respectively, because of their close structural relationship. For the calibration graph, plasma samples were spiked with increasing amounts (0.5—20 μg) of DEHP and MEHP and a constant amount (10 μg) of both internal standards using standard solutions of 0.2 μg/μl in acetonitrile. These calibration samples were taken through the extraction and chromatographic procedures described above. The peak area ratios of DEHP to di-$n$-octyl phthalate and MEHP to mono-$n$-octyl phthalate were plotted as a function of the concentration of DEHP and MEHP, and an unweighted least-squares regression analysis was performed.

*Absolute recovery*

The absolute recovery of DEHP and MEHP was determined by adding the internal standard after the extraction and comparing the peak area ratios with those of a calibration graph obtained by injecting various amounts of DEHP and MEHP to which a constant amount (10 μg) of internal standard had been added. For DEHP, the extracted samples were corrected for the contamination with DEHP originating from the extraction.

## RESULTS AND DISCUSSION

*Extraction conditions and gas chromatography*

Mono- and diesters of phthalic acid were isolated from plasma by two consecutive extractions at different pH; during an extraction at neutral pH the diesters and cholesterol were removed, while acidic monoesters were isolated after acidification of the same sample to pH 2. The use of a suspension of silicic acid in $n$-hexane for the extraction produced a reduction in the background interference peaks in plasma samples and prevented emulsification of the reagents during the extraction.

DEHP and MEHP were detected by GC using flame ionization detection. In order to improve the retention times and peak shapes, two different columns were used, both of which were kept at the same oven temperature, which allowed concurrent determination of DEHP and MEHP using two recording integrators. It is possible to separate DEHP and MEHP and their respective internal standards in one run, using a 5% SE-30 column at 200°C; however, an interfering peak elutes together with the internal standard of MEHP, and the retention time of cholesterol under these chromatographic conditions is very long.

*Esterification of MEHP*

MEHP was derivatized to its butyl ester by pyrolysis of tetrabutylammonium hydroxide in the heated inlet zone of the gas chromatograph, with methanol

catalysis. This alkylation method provides a safe alternative to derivatization with diazomethane [7, 8, 10], and does not require any preparative work. Moreover, in this procedure the diesters are not exposed to the alkylating reagent; such an exposure results in a large standard deviation for the diesters [9].

Derivatization of a compound by esterification for the purpose of analytical determination involves a chemical reaction. For this reason, the addition of a structurally related internal standard before the extraction, undergoing the same derivatization as the product to be quantitated, is necessary. In this study mono-*n*-octyl phthalate, a structural isomer of MEHP, was chosen as the internal standard.

*Sensitivity and selectivity*

Figs. 1 and 2 show representative chromatograms of plasma extracts. As shown in Fig. 1A, contamination with DEHP originating from the extraction could not be avoided entirely. Many reports have dealt with the problem of contamination with DEHP of solvents and materials used in the laboratory [11, 14]. A value of 0.37 ± 0.16 $\mu$g DEHP per 100 $\mu$l of blank sample ($n$ = 34)

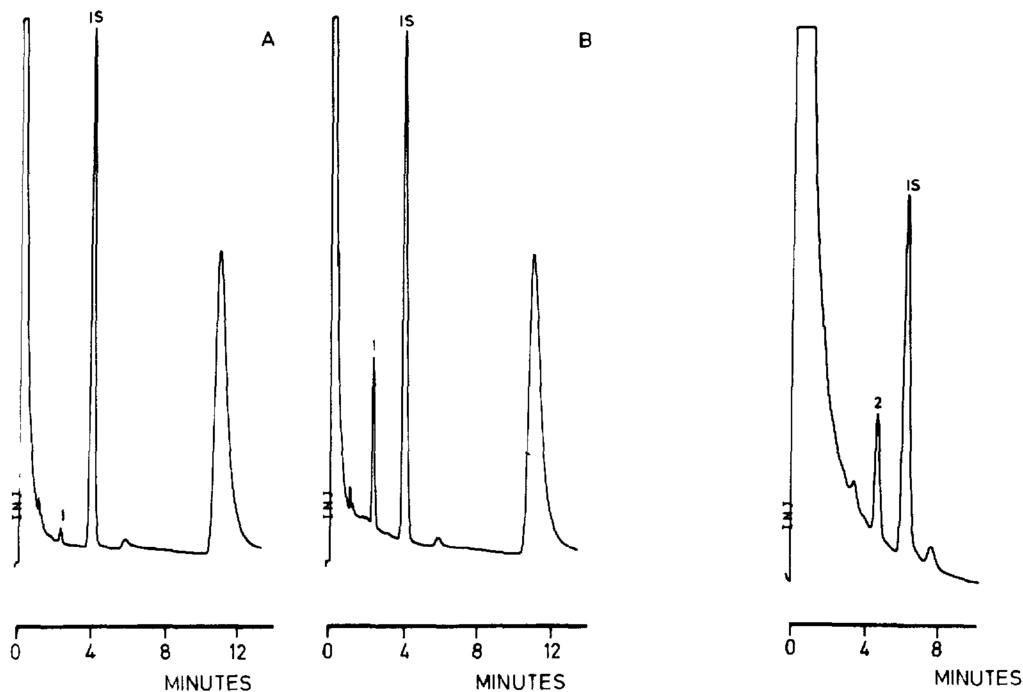


Fig. 1. Gas chromatograms of extracts of 100 $\mu$l of plasma. (A) Blank plasma sample spiked with 10 $\mu$g of di-*n*-octyl phthalate (retention time, $t_R$ = 4.15 min) as internal standard (IS); a small degree of contamination with DEHP (1) can be seen ($t_R$ = 2.46 min). (B) Plasma spiked with 2 $\mu$g of DEHP (1) ($t_R$ = 2.46 min) and 10 $\mu$g of di-*n*-octyl phthalate (IS) ($t_R$ = 4.15 min). The peak with $t_R$ = 11.30 min is cholesterol.

Fig. 2. Gas chromatogram of an extract of 100 $\mu$l of plasma spiked with 2 $\mu$g of MEHP (2) ($t_R$ = 4 63 min) and 10 $\mu$g of mono-*n*-octyl phthalate (IS) as internal standard ($t_R$ = 6 20 min).

was obtained by limiting the extraction procedure of DEHP to a single step, by using a pesticide-grade extraction solvent and by rinsing all glassware with *n*-hexane before use.

The level that still allowed the quantitation of both MEHP and DEHP was 5 μg/ml, although lower levels were detectable. By increasing the sample volume it is possible to increase the sensitivity. Extraction of 0.5 ml of plasma enabled us to detect 1.5 μg/ml of both DEHP and MEHP.

The sensitivity and selectivity of the proposed method for DEHP are similar to those reported using electron-capture detection [11]. A higher sensitivity is achieved for MEHP by this method [10].

*Precision, recovery and linearity*

The extraction gave good recoveries of both DEHP and MEHP from plasma (Table I). Plasma calibration graphs were linear from 5 to 200 μg/ml. The average slope of 34 calibration graphs constructed over a period of six months for DEHP was 0.00958 ± 0.00078 (mean ± standard deviation), with an intercept of 0.0498 ± 0.0379 and a correlation coefficient of 0.9990 ± 0.0009. For MEHP the average slope was 0.01293 ± 0.00137 with an intercept of 0.0176 ± 0.0155 and a correlation coefficient of 0.9983 ± 0.0014. The within- and between-assay accuracy and precision were acceptable (Table II).

TABLE I

ABSOLUTE RECOVERIES OF DEHP AND MEHP FROM PLASMA ($n$ = 5)

| Amount added to 100 μl of plasma (μg) | DEHP | | MEHP | |
|---|---|---|---|---|
| | Mean (%) | R.S.D. (%) | Mean (%) | R.S.D. (%) |
| 0.8 | 90.6 | 2.2 | 81.2 | 7.9 |
| 2 | 81.8 | 8.5 | 74.5 | 5.5 |
| 10 | 88.0 | 4.4 | 78.2 | 3.1 |
| 18 | 89.7 | 1.4 | 82.9 | 2.0 |

TABLE II

WITHIN- AND BETWEEN-RUN ACCURACY AND PRECISION FOR DEHP AND MEHP

| Amount added to 100 μl of plasma (μg) | DEHP | | MEHP | |
|---|---|---|---|---|
| | Relative error (%) | R.S D. (%) | Relative error (%) | R.S.D. (%) |
| *Within-run (n = 5)* | | | | |
| 0.5 | −6.4 | 10.4 | +4.6 | 15.4 |
| 1 | −3.6 | 4.8 | −3.7 | 5 2 |
| 2 | −4.5 | 4.7 | −2 0 | 4.6 |
| 10 | +3.8 | 2.5 | −4.0 | 0.9 |
| 18 | −2.2 | 4.7 | −3.1 | 1.4 |
| *Between-run (n = 38)* | | | | |
| 10 | −0.2 | 4.4 | −0.3 | 5.4 |

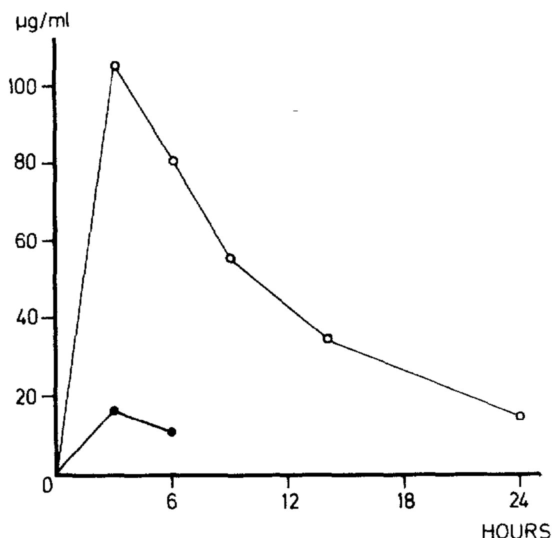


Fig. 3. Concentration—time curves of DEHP (•) and MEHP (○) in the plasma of an immature male rat after oral administration of 2.8 g/kg DEHP dissolved in corn oil (total volume: 5 ml/kg).

*Preliminary toxicokinetic investigation*

The small volume of sample required allows the study of plasma concentrations of DEHP and MEHP in rats after administration of DEHP. Fig. 3 shows plasma levels of DEHP and MEHP in a rat treated orally with 2.8 g/kg DEHP. A more detailed study of the plasma levels of DEHP and MEHP in rats will be published elsewhere [15].

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