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DETERMINATION OF DI(2-ETHYLHEXYL) PHTHALATE AND FOUR OF ITS METABOLITES IN BLOOD PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method using gas chromatography—chemical-ionization mass spectrometry has been developed for determination of the plasticizer di(2-ethylhexyl) phthalate (DEHP) in blood plasma. With selective monitoring of the protonated molecular ion, DEHP concentrations down to 75 ng per 500 μl of human plasma can be measured. Methods using electron-impact mass spectrometry with single-ion monitoring have also been developed for determination of mono(2-ethylhexyl) phthalate (MEHP) in human blood plasma, and of MEHP and other DEHP-derived metabolites in rat plasma. After extraction and derivatization with pentafluoropropanol—pentafluoropropionic anhydride, the metabolites are monitored at *m/z* 281. The precision and sensitivity of these methods indicate that they will be valuable in studies of the pharmacokinetics of DEHP and its metabolites.

INTRODUCTION

It is well recognized that blood and blood products may leach the plasticizer di(2-ethylhexyl) phthalate (DEHP) out of medical devices such as blood storage bags and extension tubing [1—3]. Consequently, patients undergoing transfusions of blood products or haemodialysis, for example, may be inadvertently

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exposed to this plasticizer. Although the acute toxicity of DEHP can be regarded as low, results of a number of studies in laboratory animals [4, 5] have pointed to possible harmful effects of long-term exposure. Repeated administration of DEHP in rats has resulted in testicular damage [6] and liver changes [7], including hepatocarcinomas [8]. These effects are probably not exerted by DEHP itself but by one or more of its metabolites [9—11]. In assessing the potential hazards to people of DEHP exposure, an understanding of the pharmacokinetics of DEHP and its metabolites in humans as well as in experimental animals is essential.

A number of analytical methods have been employed for the assay of DEHP in blood plasma. These include high-performance liquid chromatography (HPLC) [12—14] and gas chromatography (GC) with either electron-capture [15] or flame-ionization detection [16—22]. Gas chromatography coupled with mass spectrometry (GC—MS) has been used for the quantitation of DEHP in tissue extracts [23] and for the identification of DEHP in plasma samples [15, 21]. Mono(2-ethylhexyl) phthalate (MEHP), the principal metabolite of DEHP, has been determined in blood plasma by HPLC [12, 13] or by GC with flame-ionization detection [16]. GC and GC—MS have been used to identify MEHP and other metabolites of DEHP in purified extracts of urine [24, 25].

This paper describes a method for determination of DEHP in blood plasma using chemical-ionization MS with selected-ion monitoring. The paper also describes methods using electron-impact MS with single-ion monitoring for quantitation of MEHP in human plasma and for simultaneous assay of MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate and mono(5-carboxy-2-ethylpentyl) phthalate in rat plasma.

EXPERIMENTAL

Reagents and chemicals

DEHP was obtained from Fluka (Buchs, Switzerland). [^{14}C]DEHP was synthesized by treating [carbonyl-^{14}C]phthalic anhydride (Radiochemical Centre, Amersham, U.K., 48 mCi/mmol) and unlabelled phthalic anhydride with 2-ethyl-1-hexanol in the presence of *p*-toluene sulphonic acid as described by Albro et al. [24]. The specific activity of the labelled DEHP was 65 μCi/mmol. MEHP and [^{14}C]MEHP were synthesized according to the method of Chu et al. [26]. The labelled MEHP had a specific activity of 37 μCi/mmol. Mono(*n*-hexyl) phthalate (used as internal standard in the assays of DEHP-derived metabolites) was synthesized as described by Chu et al. [26], using phthalic anhydride and *n*-hexanol. The chemical identities of the synthesized unlabelled compounds were confirmed by NMR spectroscopy [^{1}H and ^{13}C spectra, JEOL (JNMFX 100)] and GC—MS (Finnigan Model 4000; Sunnyvale, CA, U.S.A.). The purity of the synthesized compounds was checked by thin-layer chromatography (TLC) and NMR spectroscopy. The radiochemical purities of [^{14}C]DEHP and [^{14}C]MEHP were higher than 98% when determined by TLC and radioactivity scanning. Mono(2-ethyl-5-hydroxyhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate and mono(5-carboxy-2-ethylpentyl) phthalate were gifts from Dr. N.E. Stjernström, Chemical Laboratory, Department of

Drugs, National Board of Health and Welfare, Uppsala, Sweden. The chemical identities of these compounds were confirmed by NMR spectroscopy and GC—MS [27]. Di(3,5,5-trimethylhexyl) phthalate (used as internal standard in the DEHP assays) was a gift from ACO (Solna, Sweden). Pentafluoropropionic anhydride (PFPA) and pentafluropropanol (PFP-OH) were obtained from Reagenta (Uppsala, Sweden). Acetonitrile, HPLC grade, was obtained from Blackford Wells (U.K.), and reagent-grade diethyl ether and *n*-hexane (distilled before use) were obtained from Merck (Darmstadt, F.R.G.).

Extraction of DEHP from human and rat plasma

A 50-μl volume of rat plasma or a 500-μl volume of human plasma was diluted with 3.0 ml of distilled water. After addition of 100 μl of internal standard solution [5.0 μg/ml di(3,5,5-trimethylhexyl) phthalate in methanol], 3.0 ml of acetonitrile and 2.0 ml of hexane, the mixture was sonicated and shaken for 15 min. After centrifugation at 1000 *g*, the hexane phase was transferred to another tube and evaporated under nitrogen at 40°C. The residue was dissolved in 50 μl of hexane and analysed by GC—MS with the spectrometer operated in the chemical-ionization mode.

Extraction of MEHP from human plasma

To 500 μl of plasma was added 1.0 ml of distilled water (pH 7.4). After addition of 100 μl of internal standard solution [2.5 μg/ml mono(*n*-hexyl) phthalate in methanol] and 3.0 ml of hexane, the mixture was shaken for 15 min. After centrifugation at 300 *g* the hexane phase was discarded. Then 8 ml of hexane and 0.2 ml of 1 *M* hydrochloric acid were added to the remaining aqueous phase, and the mixture was shaken and centrifuged. The hexane phase was transferred to another tube and evaporated under nitrogen. The remaining material was derivatized with 100 μl of PFPA—PFP-OH (2:1) for 20 min at 70°C. Excess reagent was evaporated under nitrogen. The residue was dissolved in 50 μl of hexane before analysis by GC—MS with the spectrometer operated in the electron-impact mode.

Extraction of metabolites of DEHP from rat plasma

To 50 μl of plasma was added 1.0 ml of distilled water. After addition of 100 μl of internal standard solution [2.5 μg/ml mono(*n*-hexyl) phthalate in methanol] and 0.2 ml of 1 *M* hydrochloric acid, the mixture was extracted twice with 5 ml of diethyl ether. The combined diethyl ether phases were dried over anhydrous sodium sulphate, and the remaining solvent was evaporated to dryness under nitrogen at room temperature. The residue was subjected to derivatization by the procedure described above. After evaporation of excess PFPA—PFP-OH, the remaining material was dissolved in 100 μl of hexane—toluene (2:1) before analysis.

Stability of DEHP during the work-up procedure of the MEHP assay

To investigate whether there was any conversion of DEHP into MEHP during the work-up procedure of the MEHP assay, a separate experiment was performed. In this study 0, 0.5, 1.0, 5.0, 10.0, or 40.0 μg of DEHP in 50 μl of methanol were added to glass tubes. The solutions were evaporated to dryness,

and then 500 μl of plasma were added. The plasma samples were sonicated for 3 min, left at room temperature for 15 min, and then treated by the method used for extraction of MEHP from human plasma. The extracts were derivatized with PFPA—PFP-OH and analysed for the presence of MEHP as described below.

Analysis of DEHP

During DEHP analysis the mass spectrometer was operated in the chemical-ionization mode. The samples were introduced into the gas chromatograph by the falling-needle technique. The GC analyses were performed with use of an SE-30 glass capillary column (12 m × 0.3 mm I.D.; Ultrasep®, OY Separation Research, Turku, Finland), which was directly connected to the ion source of the mass spectrometer. The flow-rate of the methane carrier gas was 1.5 ml/min. The use of methane as make-up gas resulted in an ion source pressure of 43 Pa and a pressure of $5.4 \cdot 10^{-3}$ Pa in the analyser region. The temperatures of the injector block, GC oven, transfer line, and ion source were maintained at 260, 255, 270 and 220°C, respectively. The ion source was operated at an ionizing energy of 110 eV and an emission current of 0.35 mA. The mass spectrometer was connected to an INCOS data system (Finnigan) and the instrument was set by the this system to measure *m/z* 391 and *m/z* 419, the protonated molecular ions of DEHP and the internal standard, respectively.

Analysis of metabolites of DEHP

When the metabolites of DEHP were analysed, the mass spectrometer was operated in the electron-impact mode. The samples were introduced into the gas chromatograph by the falling-needle technique and the separations were done in the same capillary column as was used in the analysis of DEHP. Helium was used as a carrier gas at a flow-rate of 1.5 ml/min. The temperatures of the injector and oven were maintained at 220 and 200°C, respectively, while those of the transfer line and ion source were 265 and 280°C, respectively. The mass spectrometer was operated at 40 eV and the data system was adjusted to record the ion *m/z* 281.

RESULTS AND DISCUSSION

Extraction and analysis of DEHP

The present method for extraction of DEHP from plasma was a slight modification of that described by Rock et al. [16]. We found that sonication prior to the extraction gave a more consistent and higher recovery than without sonication. The mean recovery as determined with ^{14}C-labelled DEHP was 93%. Since DEHP may be metabolized to MEHP by unspecific plasma esterases [28], incorrect levels of DEHP may be obtained if the plasma samples are left at room temperature for relatively long periods of time [29]. In the present extraction method the plasma samples were mixed with organic solvents within 15 min after thawing. During this time period no conversion of DEHP into MEHP seemed to occur, since plasma samples to which DEHP had been added and which were subjected to the normal extraction procedure did not contain any MEHP.

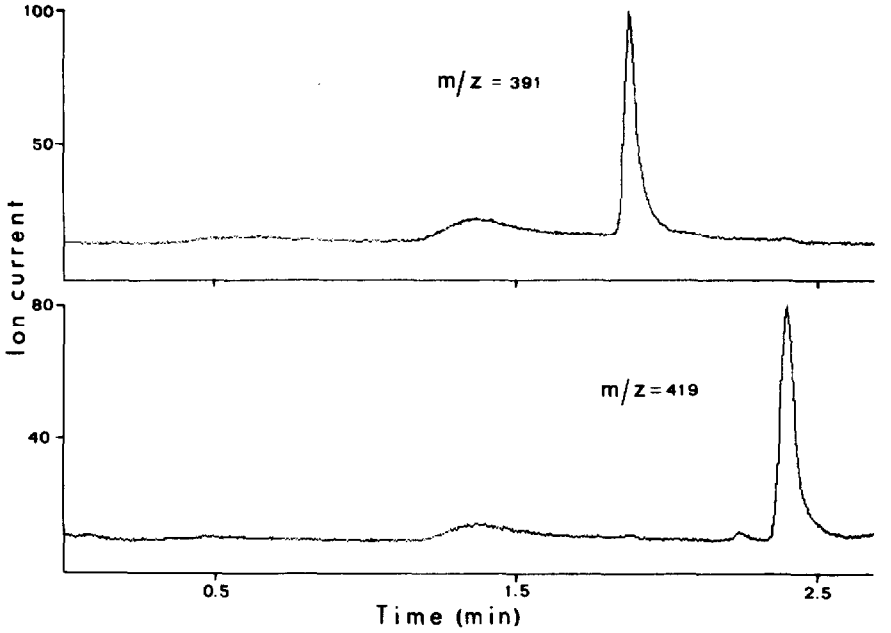

Fig. 1. Mass chromatograms showing the intensities of the protonated molecular ions of DEHP (*m/z* 391) and internal standard (*m/z* 419) in the analysis of a plasma sample from a newborn infant. The DEHP peak in the chromatogram corresponds to a concentration of 2.4 μg/ml of plasma.

All phthalic acid esters with both side-chains longer than one carbon atom have the base peak at *m/z* 149 when analysed by electron-impact MS [30]. Selective monitoring of this ion has been used to identify the occurrence of DEHP in blood plasma [15, 21]. We have found that this method is not optimal for routine monitoring of low levels of DEHP, since endogenous substances in plasma may interfere with the analysis. Chemical-ionization MS with selective monitoring of the protonated molecular ion of DEHP (*m/z* 391) appeared to be a better option in this respect. Fig. 1 shows a mass chromatogram of a plasma sample from a newborn infant. The infant had received transfusions of blood, which had been stored in poly(vinyl chloride) bags plasticized with DEHP. With the present method the coefficient of variation for the determination of 500 ng of DEHP per 0.5 ml of plasma was 3.7% (n = 10). The determination limit was 75 ng per 0.5 ml of plasma. This limit, which is similar to the lowest figures reported earlier [22, 23], is set by the levels of DEHP present in the solvents and in the general environment.

Extraction and analysis of metabolites of DEHP

The metabolic relationships between DEHP and the investigated metabolites, as proposed by Albro et al. [24], are given in Fig. 2. Hexane extraction of the primary metabolite (MEHP) from acidified human plasma gave a recovery of 80% as determined with ^{14}C-labelled MEHP. We observed that mono(2-ethyl-5-hydroxyhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate and mono(5-carboxy-2-ethylpentyl) phthalate (metabolites A, B and C, respectively) were not as readily extracted with hexane as was MEHP. Therefore, when rat plasma

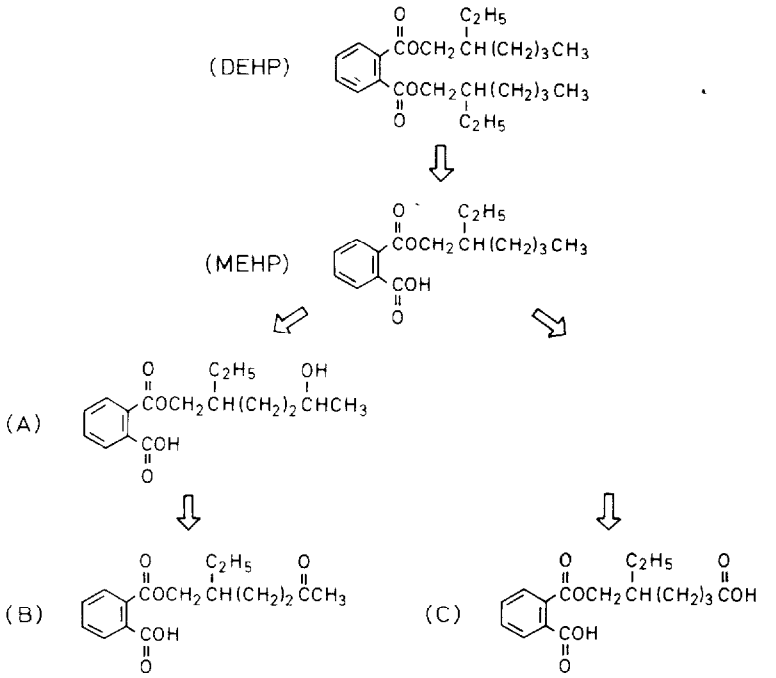

Fig. 2. Metabolic relationship between the investigated metabolites: A = mono(2-ethyl-5-hydroxyhexyl) phthalate; B = mono(2-ethyl-5-oxohexyl) phthalate; C = mono(5-carboxy-2-ethylpentyl) phthalate.

was investigated for its content of these metabolites of DEHP, diethyl ether was used as the extraction medium. The recoveries of MEHP and metabolites A, B and C were 91, 80, 87 and 57%, respectively.

The use of simultaneous perfluoroacylation and esterification of compounds or mixtures of compounds with hydroxyl and carboxyl functions has been reported [31]. Metabolites of DEHP that are derivatized with PFPA—PFP-OH all show the fragment *m/z* 281 when analysed by electron-impact MS (Table I). A large peak is also observed at *m/z* 149, which is thought to represent protonated phthalic anhydride [30]. We have found that monitoring of the metabolites at *m/z* 281 results in less interference from endogenous compounds than monitoring at *m/z* 163, which is the base peak of the methylated metabolites [32].

Fig. 3 illustrates the separation of the metabolites present in rat plasma after a 3-h intravenous infusion of 500 mg of DEHP. In the chromatogram a high peak is observed between the peaks corresponding to metabolites A and B. Since no reference compound was available, absolute identification of this extra peak was not possible. However, Harvan et al. [32] have shown that methylated metabolites of DEHP display different fragmentation patterns when analysed with chemical-ionization MS. Therefore, extracts from plasma of rats that had been treated with DEHP were methylated and the samples were subjected to selected-ion monitoring with the instrument operated in the chemical-ionization mode. The chromatogram from this analysis showed a peak with ions that were in accord with those of mono(3-carboxy-2-ethylpropyl) phthalate, as reported by Harvan et al. [32].

TABLE I

ELECTRON-IMPACT MASS SPECTRAL DATA OF METABOLITES OF DEHP

The metabolites were derivatized with a mixture of pentafluoropropionic anhydride and pentafluoropropanol as described in Experimental.

Compound*	$[M]^+$	Mean peaks m/z**				
MEHP	410 (0)	281 (78)	149 (81)	112 (75)	83 (49)	70 (100)
A	572 (0)	281 (82)	149 (100)	110 (90)	68 (67)	56 (65)
B	424 (0)	281 (24)	149 (16)	108 (18)	83 (14)	44 (100)
C	572 (0)	281 (100)	149 (71)	124 (27)	96 (29)	82 (64)
I.S.	382 (0)	281 (47)	149 (100)	92 (20)	91 (25)	57 (28)

*For A, B and C see Fig. 2; I.S. = internal standard (mono(n-hexyl) phthalate).
**The intensities of the peaks, expressed as a percentage of the base peak, are given in parentheses.

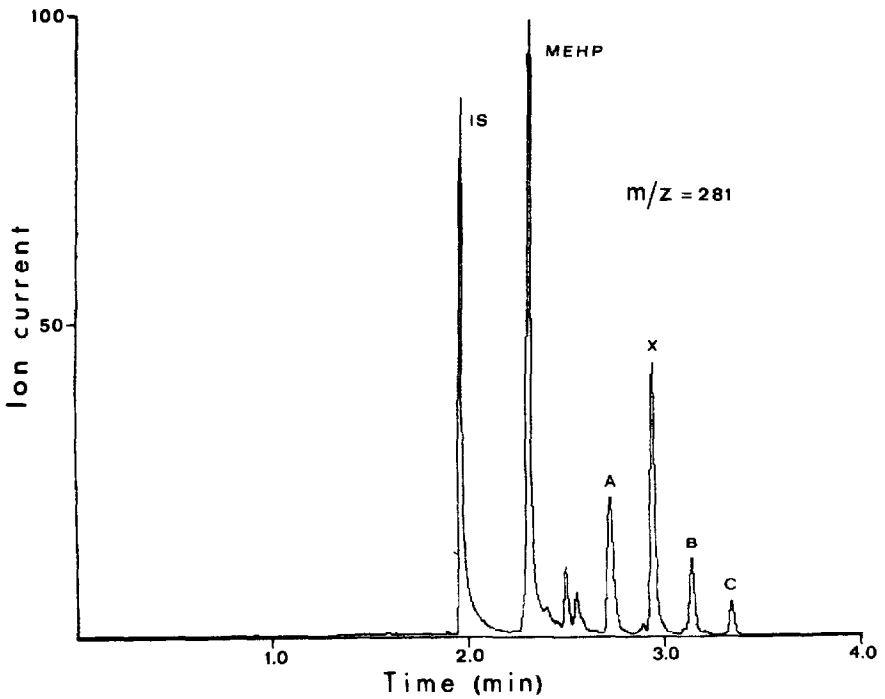

Fig. 3. Mass chromatogram showing the separation of DEHP metabolites present in rat plasma 2 h after an intravenous infusion of a DEHP emulsion. For A, B and C see Fig. 2. The peaks of MEHP and the metabolites A, B and C correspond to concentrations of 33.8, 6.2, 3.8 and 2.5 μg/ml of plasma, respectively. X has tentatively been identified as mono(3-carboxy-2-ethylpropyl) phthalate.

The determination limit of MEHP when extracted from human plasma with hexane was 10 ng/ml, and the precision of the method, at a concentration of 100 ng per 500 μl, was 3.8% (n = 10). Table II shows the coefficients of variation and the determination limits for the DEHP-derived metabolites when extracted from rat plasma with diethyl ether. The low determination

TABLE II

DETERMINATION LIMITS AND PRECISIONS OF THE ASSAY OF DEHP-DERIVED METABOLITES

Compound*	Determination limit (ng/ml)	Coefficient of variation (%)** (at plasma concentration)
MEHP	2.0	3.5 (120 ng per 50 μl)
A	2.6	7.9 (10 ng per 50 μl)
B	2.0	6.2 (10 ng per 50 μl)
C	2.3	8.7 (10 ng per 50 μl)

*For A, B and C see Fig. 2.
**$n = 10$.

limits obtained with these methods indicate that the methods will be valuable for studying the pharmacokinetics of these compounds in different mammalian species.

Stability of DEHP during the work-up procedure of the MEHP assay

No MEHP was detected in plasma samples to which up to 40 μg of DEHP had been added. This suggests that no conversion of DEHP into MEHP occurs during the extraction and derivatization procedure and that the MEHP found in plasma originates from in vivo metabolism of DEHP.

CONCLUSION

The present results show that GC coupled with chemical-ionization MS, with selected monitoring of the protonated molecular ion, is a useful method for routine determination of DEHP in blood plasma. It is also apparent that electron-impact MS, with monitoring of the ion *m/z* 281, is a convenient method for simultaneous determination of perfluoroacetylated/esterified metabolites of DEHP.

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