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Chromatographic fractionation and analysis by mass spectrometry of conjugated metabolites of bis(2-ethylhexyl)phthalate in urine

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Abstract

Mono(2-ethylhexyl)phthalate (MEHP), the primary metabolite of the plasticizer bis(2-ethylhexyl)phthalate (DEHP), was given to guinea pigs and mice and the methods for the isolation, separation and analysis of its metabolites in urine were developed. Following solid-phase extraction with octadecylsilane-bonded silica, individual metabolites were purified and separated using a combination of ion-exchange chromatography on lipophilic gels and reversed-phase high-performance liquid chromatography. Analysis of intact conjugates, as well as nonconjugated metabolites, was performed by fast atom bombardment mass spectrometry (FAB–MS) and, after derivatization, by gas chromatography–mass spectrometry. Enzymatic methods were used for further characterization. The study confirms glucuronidation as the major conjugation pathway for MEHP in the investigated species. Although less important quantitatively, glucosidation is shown to be an alternative conjugation pathway in mice. The methods developed were applied to a sample of urine from a hyperbilirubinemic newborn infant subjected to DEHP-exposure in conjunction with an exchange transfusion. It was demonstrated that metabolites of DEHP were excreted in amounts which could be analyzed by FAB–MS.

Keywords: Mono(2-ethylhexyl)phthalate; Bis(2-ethylhexyl)phthalate

1. Introduction

Bis(2-ethylhexyl)phthalate (DEHP)[1] is a plasticizer that is ubiquitous in the general environment [1,2]. It is a constituent in medical devices such as blood storage bags and medical tubing. Leakage of this plasticizer lead to significant exposure in certain groups of patients [3,4], which principally could result in toxic effects [5–9]. Due to exposure of humans to DEHP it is important to have knowledge of its metabolism and to develop methods for biological monitoring.

The first step in the degradation of DEHP, which is common to all investigated species, is the hydrolysis to mono(2-ethylhexyl)phthalate (MEHP) [10–14]. Further metabolism is species specific. Man, monkey, guinea pig and mouse excrete MEHP and its products mainly as conjugates with

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[1] The accepted abbreviation for this compound has been retained, although it does not reflect the correct systematic name according to IUPAC.

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glucuronic acid [10,11,13,14]. Analysis of such conjugates has mainly been performed after enzymatic hydrolysis [10,13], but positive and negative chemical ionization (CI) mass spectra of the glucuronide of MEHP have also been recorded [10].

The aim of the present investigation was to develop methods for the analysis of conjugated and unconjugated metabolites of DEHP/MEHP in urine. Since the same metabolites are formed after administration of MEHP and DEHP [5,12], the former compound was used. Urine was collected from guinea pigs and mice, that had been given MEHP. After extraction and chromatography, the material was analysed by fast atom bombardment mass spectrometry (FAB–MS) and gas chromatography–mass spectrometry (GC–MS), using electron-impact (EI) ionization. The methods developed were applied to the analysis of urine from a hyperbilirubinemic infant subjected to an exchange transfusion.

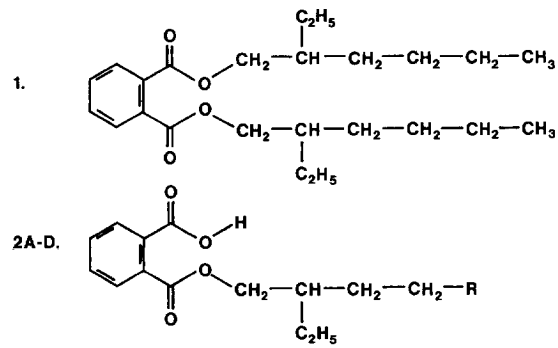

Fig. 1. Structures of DEHP and the metabolites of DEHP available to us as standards. The designations of the metabolites by Roman numerals are as used by Albro et al. [11,12]. 1=Bis(2-ethylhexyl)phthalate (DEHP); 2A=Mono(2-ethylhexyl)phthalate (MEHP), R=-CH_2CH_3, metabolite XI; 2B=Mono(2-ethyl-5-hydroxyhexyl)phthalate (MEHP-alc), R=-$CHOHCH_3$, metabolite IX; 2C=Mono(2-ethyl-5-oxohexyl)phthalate (MEHP-ket), R=-$COCH_3$, metabolite VI; 2D=Mono(2-ethyl-5-carboxypentyl)-phthalate (MEHP-diacid), R=-CH_2COOH, metabolite V.

2. Experimental

2.1. Chemicals

Solvents (Merck, Darmstadt, Germany) were of analytical grade and redistilled in an all-glass apparatus with a spinning-band column before use. Water was deionized and purified with a Milli-Q cartridge (Millipore, Bedford, MA, USA). All other chemicals (Merck) were of analytical grade or better and were used as supplied if not otherwise indicated. Glassware was cleaned in an ultrasonic bath.

Unlabelled MEHP, mono(2-ethyl-5-hydroxyhexyl)phthalate (MEHP-alc), mono(2-ethyl-5-oxohexyl)phthalate (MEHP-ket) and mono(2-ethyl-5-carboxypentyl)phthalate (MEHP-diacid) were those used in a previous study [15]. The structures are shown in Fig. 1. [^{14}C]Mono(2-ethylhexyl)phthalate ([^{14}C]MEHP) was synthesized from [carbonyl-^{14}C]phthalic anhydride (500 μCi, 58 mCi/mmol; Amersham, UK) and 2-ethyl-1-hexanol (0.02 mmol; Berol Kemi, Stenungsund, Sweden) by heating the two compounds in 100 μl of toluene at 110°C for 6 h. The reaction mixture was purified by preparative TLC (vide infra). TLC showed that the isolated compound (414 μCi) was radiochemically pure and had the same R_f value as unlabelled MEHP.

Helix Pomatia digestive juice was from l'Industrie Biologique Francaise (Clichy, France). α-Glucosidase (from bakers yeast) and β-glucosidase (from almonds) were obtained from Sigma (St. Louis, MO, USA).

2.2. Gels and columns

Octadecylsilane-bonded silica, Sepralyte (Analytichem, Harbor City, CA, USA) was washed with methanol (10 ml) and water (10 ml) prior to use [16]. SP-Sephadex C-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden) and Lipidex-DEAP was from Packard Instruments Co. (Downers Grove, IL, USA). These gels were washed with aqueous ethanol [17] and stored in methanol at 4°C.

Gel beds were prepared in glass columns of about 4 mm I.D., with a gauze-covered valve of PTFE to hold the bed. The columns had a solvent reservoir at the top and, when necessary, a nitrogen pressure could be applied.

2.3. Thin-layer chromatography

Silica gel plates SiO_2 60, F_{254}, 0.25 mm (Merck) were used for TLC with the solvent system 1-butanol–acetic acid–water (10:1:2, v/v). Spots were

viewed under UV light (254 nm) and visualized by spraying with 50% sulphuric acid or chromic acid followed by heating. Radioactive spots were visualized by autoradiography (X-ray film D7pDW, Agfa-Gevaert, Belgium). Silica gel plates as above but with concentration-zone were used for preparative TLC with dichloromethane–ethylacetate (1:1, v/v) as the mobile phase.

2.4. High-performance liquid chromatography (HPLC)

The instrument used for HPLC system 1 consisted of two LDC Constametric III pumps, an LDC gradient Master (Laboratory Data Control, Milton Roy, Riviera Beach, FL, USA) and a Model 7125 injector (Rheodyne, Cotati, CA, USA) with a 1-ml loop. The column system comprised an RP-18 guard cartridge (15 mm × 3.9 mm I.D.; Brownlee Laboratories, Santa Clara, CA, USA) and a semipreparative μBondapak C_{18} stainless steel column (15 cm × 19 mm I.D., particle size 10 μm; Waters, Milford, MA, USA).

The samples were injected in 50–200 μl of 50% aq. acetonitrile and eluted with a linear gradient of 30–60% aqueous acetonitrile, containing 0.1% trifluoroacetic acid, over a period of 30 min and held at the final conditions for 20 min. The flow-rate was 5 ml min^{-1}. The UV absorption at 254 nm and the radioactivity were monitored continuously, using an LDC UV Monitor III, Model 1203 (Laboratory Data Control) and a Trace 7140 radioactivity flow monitor (Packard) utilizing heterogeneous detection mode (europium activated calcium fluoride), respectively. Fractions were collected with a fraction collector Model 201 from Gilson (Villiers Le Bel, France).

HPLC system 2 [18] was used with a column system of a Guard-Pak with a μBondapak C_{18} cartridge and a Nova-Pak C_{18} Radial Pak cartridge (10 cm × 8 mm I.D., particle size 4 μm) in a Z-module (Waters). The samples were injected in 50 μl of 50% aqueous acetonitrile and eluted from the column with 30% or 45% aqueous acetonitrile, containing 0.1% trifluoroacetic acid, with a flow-rate of 1 ml min^{-1}.

Appropriate fractions from each HPLC step were combined and concentrated by evaporating the acetonitrile under a stream of nitrogen and were finally lyophilized. Aliquots were taken for liquid scintillation counting.

2.5. Determination of radioactivity

Radioactivity was determined in an LKB 1211 Minibeta liquid scintillation counter (LKB-Wallac, Bromma, Sweden), using Insta-Gel (Packard) as scintillation liquid.

2.6. Fast atom bombardment–mass spectrometry

FAB–MS was performed with a VG 7070E double focusing mass spectrometer equipped with a FAB ion source and an Ion Tech atom gun (VG Analytical, Manchester, UK). An aliquot of the samples, usually 1–5 μg, dissolved in methanol, was applied under a slight stream of nitrogen to the FAB target already covered with the glycerol matrix. The ion source was operated at 6 kV and xenon having 8 keV energy was used in the primary ionization beam. Normal spectra were recorded between m/z 800 and m/z 80 by the VG 11-250 data system at a scan rate of 10 s per decade and a resolution of 1000 (5% valley).

Daughter-ion spectra of quasimolecular ions were recorded by performing linked scans keeping the ratio between the magnetic and the electrostatic fields (B/E) constant [19]. The slits were usually opened to a resolution of about 500 (5% valley) to increase the ion transmission.

Accurate mass determinations were made using accelerating voltage scans by the software peak-matching technique or magnetic scanning using the peaks from glycerol as both the primary and the secondary reference. The resolution used was 2000–5000 (5% valley).

2.7. Gas chromatography

A Vega 6000 GC (Farmitalia Carlo Erba, Milano, Italy) was used with an SP 4270 integrator (Spectra Physics, Darmstadt, Germany). The column was a 25 m × 0.32 mm I.D. fused-silica capillary coated with cross-linked methyl-silicone (0.25 μm film thickness; Quadrex Corp., New Haven, CT, USA). Helium was used as the carrier gas at 50–100 kPa. The samples were injected using the on-column

technique as methyl ester trimethylsilyl (TMS) ether derivatives [20] in 0.5–1 μl of hexane at 60 °C. The temperature was increased to 190°C at a rate of 25°C min^{-1} and then to 300°C at a rate of 2°C min^{-1}. Detection was by a flame ionization detector.

2.8. Gas chromatography–mass spectrometry

GC–MS was performed on the VG 7070E instrument equipped with the combined EI/CI ion source and a Dani 3800 gas chromatograph. The same type of capillary column was used as in the GC analysis. The column was either kept at 270°C (isothermal conditions) or after 30 s taken from 180°C to 270°C at a rate of 10°C min^{-1} and kept under these final conditions for 10 min (temperature programmed conditions). The column was directly connected and extended into the ion source. An all-glass falling needle injection system was used. The ionization energy was 70 eV and the trap current 200 μA. Spectra were recorded by repetitive magnetic scanning between *m/z* 800 and *m/z* 45 by the data system at a scan-rate of 1.5 s per decade and a 0.5-s inter-scan delay. The accelerating voltage was 6 kV and the resolution was about 1000 (5% valley).

2.9. Animal experiments

Male guinea pigs (Dunkin Hartley), weighing 400–450 g, were obtained from Sahlins Försöksdjursfarm (Malmö, Sweden). Mice were obtained from Alab (Sollentuna, Sweden). [^{14}C]MEHP was diluted with unlabelled MEHP to give a specific activity of 2.7 μCi $mmol^{-1}$ and each guinea pig was given a single oral dose of 400 mg of this compound, in corn oil, per kg body weight. In experiments with mice, [^{14}C]MEHP was diluted to a specific activity of 6.1 μCi $mmol^{-1}$ and eleven mice were given single oral doses of 400 mg of this compound, in corn oil, per kg body weight. Urine was collected on ice over 48 h. The pH was adjusted to ca. 5 using 0.1 *M* aqueous hydrochloric acid and the urine was stored at −20°C until analyzed.

2.10. Human urine

Urine was collected for 8 h from a newborn infant after an exchange transfusion performed due to hyperbilirubinemia. The hyperbilirubinemia was caused by Rh-immunization. The infant, a girl, had been born after 39 weeks of gestation with a birth weight of 3490 g. The exchange transfusion was performed on day 3. The serum bilirubin level before the transfusion was 310 μmol l^{-1}. During the transfusion, which was performed as described earlier [4], 170 ml of blood per kg body weight was exchanged. The serum bilirubin level after the exchange transfusion was 140 μmol l^{-1}. The urine was stored at −20 °C until analyzed.

2.11. Analytical procedure

Isolation and purification of metabolites in urine

Samples of urine (5–10 ml) were extracted using 100 mg of octadecylsilane-bonded silica (Sepralyte). After a wash with 10 ml of water, extracted material was eluted with 10 ml of methanol. The eluate was reduced to ca. 3.5 ml under a stream of nitrogen, diluted with ca. 1.5 ml of water and applied to a column of 0.6 ml of SP-Sephadex C-25 (in 70% aqueous methanol) in the hydrogen form. The column was washed with 70% aq. methanol until 3 × 5 ml was collected. Sorbed material was eluted with 2 × 5 ml of 0.3 *M* ammonia in 70% aqueous methanol.

The first two 5-ml fractions from the cation exchanger (containing compounds not sorbed) were combined and evaporated using a Rotavapor. The residue was dissolved in 5 ml of 70% aqueous ethanol and fractionated on a 3-ml column of Lipidex-DEAP (in 70% aqueous ethanol) in the acetate form. A series of displacers of increasing strength were used and the fractions were collected as shown in Table 1.

When radioactive material was analyzed, aliquots of 1–5% were taken for liquid scintillation counting. The fractions containing radioactivity eluted with one displacer were combined. The ethanol was evaporated on a Rotavapor and the remaining liquid was removed by lyophilization. The combined fractions were either analyzed directly with FAB–MS and/or further purified using HPLC system 1. Final purification was performed using HPLC system 2.

During development of this procedure, methanol was used instead of ethanol, and the concentrations

Table 1
Displacers used for fractionation on Lipidex-DEAP anion exchanger

Fraction	Displacer	Volume collected (ml)	Class of compounds expected to be eluted, cf. Ref. [21]
1	70% Aqueous ethanol	15	Neutrals,
2		5	phenols
3	0.05 *M* acetic acid in 70% aqueous ethanol	5	Weakly acidic, stronger than phenols
4	0.25 *M* Formic acid	5	Glucuronides
5	in 70% aqueous ethanol	5	
6		5	
7		5	
8	0.3 *M* Acetic acid–potassium	5	N-Acetylcysteine
9	acetate in 70% aqueous methanol	5	conjugates
10	(apparent pH = 6.0)	5	(mercapturic acids)
11		5	
12	0.3 *M* Acetic acid–potassium	5	Sulphates
13	hydroxide in 70% aqueous	5	
14	methanol	5	
15	(apparent pH = 10)	5	

and volumes of acetic acid and formic acid were also varied.

Enzyme hydrolyses were performed by incubating 0.1–1.0 mg of purified material with 300 μl of the digestive juice of *Helix Pomatia* in 200 m*M* aqueous sodium acetate buffer, pH 4.5 (36°C, 24 h), with 50 U α-glucosidase in 50 m*M* aqueous potassium dihydrogen phosphate buffer, pH 6.0 (25°C, 16 h) or with 50 U β-glucosidase in 50 m*M* aqueous sodium acetate buffer, pH 5.0 (25°C, 16 h). The incubation volume was 4 ml in all cases. Incubations without enzyme served as controls. At the end of the incubations, the solutions were extracted with Sepralyte as described for urine and extracted material was eluted with methanol. The methanol was evaporated on a Rotavapor and the residues were analyzed using HPLC system 2.

3. Results and discussion

3.1. Extraction and chromatography

The urine from MEHP-treated animals was subjected to solid-phase extraction using Sepralyte. Extracted material was recovered with methanol. This procedure gave a recovery of 83–103% of the ^{14}C in the urine from guinea pigs and slightly less, 74–78%, from mice. Passing the urine a second time through Sepralyte did not increase the extraction recovery significantly. This indicated that the non-extracted ^{14}C-labelled material is too polar to be extracted by Sepralyte.

The cation exchanger (SP-Sephadex C-25) sorbs material containing an amino group [18,21]. No radioactive material was sorbed, when the Sepralyte extracts were applied to the cation exchanger. This clearly indicates the absence of metabolites having an amino group in accordance with the accepted metabolic pathway of DEHP/MEHP [14]. Regardless of this result, the cation exchanger was included in the purification scheme, in order to remove cationic material. This is known to facilitate later steps in the purification procedure [21].

A group fractionation according to the acidity of the analytes, using the lipophilic anion exchanger Lipidex-DEAP, was the next step. This approach was originally designed for group separations of conjugates of steroids and bile acids [22,23], but has also been applied to the analysis of xenobiotics and their metabolites [18,24–26]. Since the metabolites from guinea pigs were quantitatively sorbed by the anion

exchanger, it was concluded that they were all acids. They were eluted with 0.25 *M* formic acid in 70% aqueous methanol or ethanol. In contrast, not all metabolites from mice were sorbed by the anion exchanger. A neutral fraction of ca. 3% of the applied radioactivity was obtained and a weakly acidic fraction was collected using 0.25 *M* acetic acid in 70% aqueous methanol. There was an overlap between the latter material and that eluted with formic acid in 70% aqueous methanol and this mode of fractionation was used only during the development of the procedure. However, the weakly acidic fraction was of special interest since it contained one of the new metabolites found in urine from mice (vide infra). The formic acid displacer also eluted the majority of metabolites from mice [27]. The stronger displacers (see Table 1) did not release any further radioactivity from samples of either guinea pigs or mice. The described fractionation procedure has been performed also using an automated sample processor [28].

Fractions obtained with the same displacer were normally combined and the solvent was removed. They were subjected to semipreparative reversed-phase HPLC on the μBondapak C_{18} column (HPLC system 1). UV absorbance and radioactivity were monitored. The UV trace of a representative sample from a guinea pig is shown in Fig. 2. Appropriate fractions were further separated and very high resolving conditions were obtained, utilizing the Nova-Pak C_{18} column and the radial compression technique with the Z-module (HPLC system 2).

The above procedure was used to obtain pure glucuronic acid conjugates of MEHP, MEHP-alc and MEHP-ket. The purity of the compounds was established by use of HPLC, TLC and FAB–MS. It was determined to be better than 98%. It was possible to obtain 10–15 mg from the urine of guinea pigs, of which the MEHP-glucuronide was the dominating metabolite [10,27]. The mice have an even distribution of the glucuronides of MEHP, MEHP-alc and MEHP-ket in urine [10,27], which can be used as a source for the latter two compounds.

The glucuronides of MEHP, MEHP-alc and MEHP-ket were treated with the digestive juice from *Helix Pomatia*. This liberated the expected aglycones quantitatively Table 2, while control incubations left the conjugates intact. Interestingly, the compound in peak nine of the HPLC separation of MEHP metabolites in urine from guinea pigs, considered to represent a glucuronide of MEHP by FAB–MS (vide infra and Table 2), was resistant to this enzymatic treatment. We have tentatively assigned the structure of this compound to be a rearranged glucuronide, formed by intramolecular transacylation. This rearrangement is known to occur with ester glucuro-

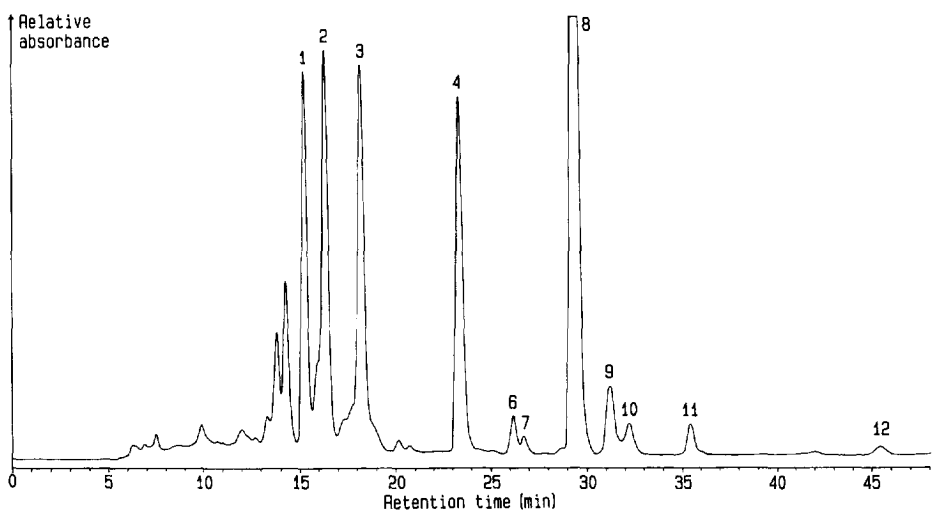

Fig. 2. The UV trace (254 nm) obtained in the HPLC analysis (system 1) of MEHP metabolites in the urine from a representative sample of a guinea pig. The metabolites appeared in the 'glucuronide fraction' from the anion-exchange chromatography. Peaks are numbered as in Table 2.

Table 2
Results of FAB–MS and enzymatic treatment of metabolites of MEHP appearing in the fraction eluted with 0.25 *M* formic acid in 70% aqueous methanol from Lipidex-DEAP anion exchanger.

Peak on HPLC system 1[a]	Peak on HPLC system 2	Important anions by negative FAB–MS (*m/z*)	Proposed Metabolite	Product after *Helix Pomatia* treatment
1	A	469 (M-1), 293, 193	Glucuronide of MEHP-alc	MEHP-alc[b]
2	A (major)	467 (M-1), 291, 193	Glucuronide of MEHP-ket	MEHP-ket[b]
	B	469 (M-1), 293, 193	Glucuronide of MEHP-alcohol (isomer to 1A and 3B)	
	C	513 (M-1), 337	Unknown glucuronide, cf. 6C	
3	A (major)	279 (M-1)	Probably 2-ethyl-3-carboxypropyl phthalate	
	B	469 (M-1), 293	Glucuronide of MEHP-alcohol (isomer to 1A and 2B)	
4	A	307 (M-1)	MEHP-diacid[b]	
5	A	293 (M-1)	MEHP-alc[b,c]	
6	A (major)	291 (M-1)	MEHP-ket[b]	
	B	293 (M-1)	MEHP-alcohol[d] (isomer to 5A and 7A)	
	C	337 (M-1)	Unknown, cf. 2C	
7	A	293 (M-1)	MEHP-alcohol (isomer to 5A and 6B)	
8	A	453 (M-1), 277, 193	Glucuronide of MEHP	MEHP[b]
9	A	453 (M-1), 277, 193 *m/z* 193 much less intense than in 8A	Glucuronide of MEHP, probably rearranged[e]	Resistant
10	A	321 (M-1)	Methyl ester of MEHP-diacid	
11	A	468 (M), 277 (Positive ions: 469 (M+1), 279)	Methyl ester of the glucuronide of MEHP	Resistant
12	A	277 (M-1)	MEHP[b]	

[a]Numbered as in Fig. 2 (and Figs. 1 and 2 in ref. [27]).
[b]Identical with reference compound, cf. Fig. 1.
[c]Not detected in samples from guinea pigs.
[d]Not detected in samples from mice.
[e]See Section 3

nides (cf. [29]) and the product no longer has the β-glucosidic bond necessary for enzymatic release. It has been shown that the acyl moiety originally attached to C-1 on the glucuronic acid moiety migrates around the pyranose ring (C-2–C-4) under mild alkaline (pH 7–9) conditions [30,31]. This may also occur in vivo [30,32,33], which is of importance if analysis is performed after enzymatic release.

3.2. Mass spectrometry of metabolites of DEHP

The conjugated and unconjugated metabolites of DEHP/MEHP found in the fractions eluted from the anion-exchange column with 0.25 *M* formic acid in 70% aqueous methanol or ethanol, "the glucuronide fractions", were subjected to analysis with FAB–MS. It was found that all these compounds ionized well under FAB conditions [27] and their identities are shown in Table 2.

The unconjugated metabolites revealed only quasimolecular ions. For the glucuronides, the only fragments identified were due to loss of the glucuronic acid moiety (−176 amu both in positive- and negative-ion mode) and an ion at *m/z* 193 present only in negative-ion mode and interpreted as the glucuronide anion. Spectra of negative ions were more informative due to relatively higher yields of analyte-related ions as compared to matrix-related ions. The negative-ion FAB mass spectrum of the glucuronide of MEHP is shown in Fig. 3.

Preparation of the methyl ester TMS ether derivatives of the major glucuronides gave good chromato-

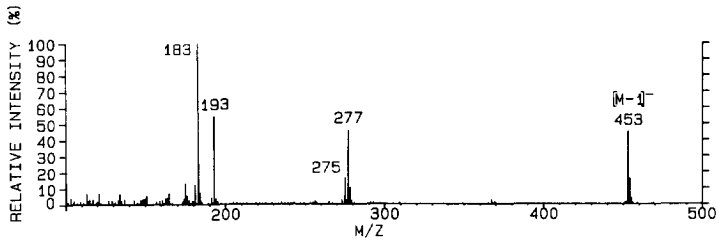

Fig. 3. Negative-ion FAB mass spectrum of the glucuronide of MEHP. The ion at m/z 453 is the quasimolecular anion, the ion at m/z 277 represents the aglycone and the ion at m/z 193 is the glucuronide anion. The ions of m/z 275 and 183 originate from the glycerol matrix.

graphic properties on the methyl-silicone column utilized for GC. All three glucuronides were therefore subjected to GC–MS studies. It was found that EI ionization gave spectra complementary to those obtained by FAB–MS. The spectra were dominanted by ions derived from the glucuronic acid moiety (m/z 406, 317, 275, 217 and 204) [34], but ions derived from the aglycone were also found, such as m/z 163 and 149 [35]. Fig. 4 shows the EI mass spectrum of the methyl ester TMS ether derivative of the glucuronic acid conjugate of MEHP.

3.3. Artefact formation on the anion exchanger detected by FAB–MS

Negative-ion FAB–MS of the material in peak 11 obtained after HPLC separation of MEHP metabolites in urine from guinea pigs, gave a strong ion at m/z 468 [27]. The corresponding ion in positive-ion mode was m/z 469. Further studies of this compound were performed with GC–MS and FAB–MS, including accurate mass determinations. These studies established the compound in peak 11 to be the methyl ester of the glucuronide of MEHP [36]. The anion at m/z 468 is a radical molecular anion, M^-, a rather unusual ion to be formed by FAB ionization. The cation at m/z 469 is the normally generated quasimolecular cation, $[M+H]^+$. The compound is neutral, but despite that fact, is recovered from the anion-exchange column in the glucuronide fraction using 0.25 *M* formic acid in 70% aqueous methanol. It must therefore have been formed on the column, thus being an artefact. In fact, we have found that the material in peak 10 originally identified as mono(2-ethyl-5-carboxy-4-oxopentyl) phthalate [27], is a monomethylated MEHP-diacid. This was proven by treating the material in peak 10 as well as MEHP-diacid, with diazomethane. Both derivatives had the same retention time when compared either by HPLC or GC. This type of artefact formation related to the use of the anion exchanger Lipidex-DEAP has not been reported previously. However, most previous fractionations have been made prior to methylation and analysis by GC–MS and, under these conditions, this type of artefact formation would not be observed. The details of the structural assignment of

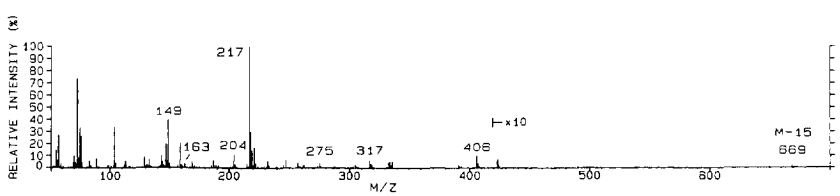

Fig. 4. EI mass spectrum of the methyl ester TMS ether of the glucuronide of MEHP. The ions at m/z 406, 317, 275, 217 and 204 are indicative of the glucuronic acid moiety and m/z 163 and 149 are indicative of the aglycone moiety. It should be noted that the $[M-15]^+$ ion at m/z 669 is detected.

the compound in peak 11 as well as a discussion of the unexpected formation of radical molecular anions under FAB conditions have been reported separately [36]. It was later shown that this type of artefact formation was absent when the anion-exchange chromatography on Lipidex-DEAP was performed in aqueous ethanol solutions. No ethyl analogues were produced and ethanol is thus the solvent recommended to be used when preparing the displacers.

3.4. Glucosidation as a conjugation pathway for metabolites of DEHP

In the analysis of mouse urine, minor metabolites were found in the neutral and in a weakly acidic fraction, the latter obtained by elution with 0.25 *M* acetic acid in 70% aqueous methanol. A total of three new metabolites were isolated from these fractions by HPLC. The chromatographic characteristics of these compounds indicated polarities similar to those of glucuronides. FAB–MS indicated the presence of a conjugated hexose (loss of 162/163 amu from the molecular mass related ions). Two of the metabolites, isolated from the neutral fraction, revealed radical molecular ions under negative-ion mode FAB conditions, which has been discussed in detail in [36]. The radical molecular ions showed the loss of 163 amu. When the two metabolites were treated with β-glucosidase, MEHP-alc and MEHP-ket were liberated from the corresponding conjugates. MEHP-diacid was obtained from the metabolite isolated from the weakly acidic fraction. Treatment with α-glucosidase and control incubations left the conjugates intact. Therefore, these metabolites were considered to be conjugates of β-glucose, with the glucose in ester linkage to the phthalic acid moiety. This was further confirmed by analysis of the methyl ester TMS ethers by GC–MS showing expected fragmentation (*m/z* 361, 319, 217 and 204) [37], and daughter-ion analysis under FAB conditions confirmed the origin of the 162/163 amu loss from the molecular mass related ions.

Glucosidation is a newly discovered conjugation pathway of DEHP metabolites and has been communicated briefly [38]. However, only a minor fraction of the administered dose (approximately 3%) was found in the urine as β-glucose conjugates. We have not been able to find any evidence for the presence of β-glucose conjugates in urine from guinea pigs or in the human urine sample analyzed.

3.5. FAB–MS of DEHP metabolites in human urine

We have earlier reported on the exposure and dispositions of MEHP and DEHP in newborn infants subjected to exchange transfusions [4,39]. The relatively long elimination half-life of DEHP found, 10 h, indicated that there is a rather long exposure to the potentially toxic metabolite MEHP following an exchange transfusion [4].

In the present study, a detailed analysis was performed of conjugated and unconjugated metabolites of DEHP excreted in the urine from a newborn infant, who had undergone an exchange transfusion due to hyperbilirubinemia. The urinary metabolites were fractionated and purified according to method described in Section 2.11. The "glucuronide fraction" from anion-exchange chromatography (obtained by use of 0.25 *M* formic acid in 70% aqueous ethanol) was subjected to FAB–MS analysis. The negative-ion FAB mass spectrum was dominated by ions derived from several metabolites of DEHP (Fig. 5), but no conjugated MEHP was found. In comparison with data from earlier studies in adults [11,13], the present results indicate that there is a somewhat higher capacity for oxidation of MEHP in newborns.

The neutral, the weakly acidic and the "glucuronide" fractions were subjected to HPLC separation. No material that could be related to DEHP was found in the neutral and the weakly acidic fractions, when UV absorbing peaks were collected and studied by FAB–MS and/or GC–MS.

4. Conclusions

Solid-phase extraction, now an established method for the extraction of intact conjugates in urine and other body fluids, was found suitable for metabolites of DEHP/MEHP. Lipophilic ion exchangers were used to fractionate the metabolites according to charge and acidity. This fractionation was essential

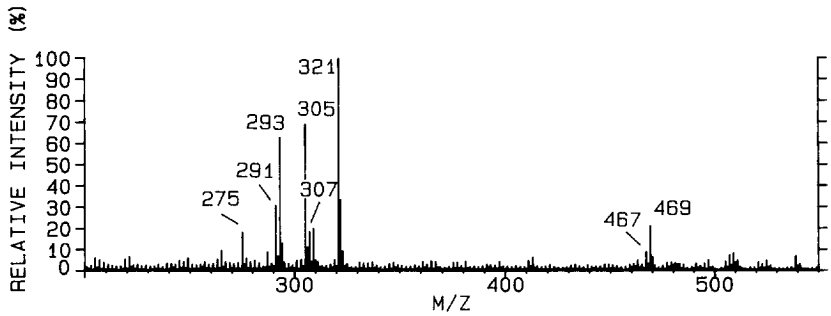

Fig. 5. Negative-ion FAB mass spectrum of the 'glucuronide fraction' from urine of a newborn infant, who had undergone an exchange transfusion due to hyperbilirubinemia. The spectrum is interpreted as follows: *m/z* 469 is the quasimolecular anion from the glucuronide of MEHP-alc, *m/z* 467 is from the glucuronide of MEHP-ket, *m/z* 321 is from the monomethyl ester of MEHP-diacid, *m/z* 307 is from MEHP-diacid, *m/z* 305 is unknown, *m/z* 293 is from MEHP-alc or is a fragment of *m/z* 469, *m/z* 291 is from MEHP-ket or is a fragment of *m/z* 467 and *m/z* 275 originates from the glycerol matrix.

Table 3
Summary of the metabolites of DEHP/MEHP and their mode of conjugation found in the urine of guinea pigs, mice and a newborn infant who had undergone an exchange transfusion

Metabolite	Mode of conjugation						
	Unconjugated			Glucuronic acid conjugate			β -Glucose conjugate
	Guinea pigs	Mice	Infant	Guinea pigs	Mice	Infant	Mice
MEHP	X	X		X	X		
MEHP-alc and isomers	X	X		X	X	X	X
MEHP-ket and isomers	X	X		X	X	X	X
MEHP-diacid	X	X	X				X
2-Ethyl-3-carboxypropylphthalate (tentative)	X	X					

in the purification of minor metabolites like the β-glucosides found in urine from mice. FAB was demonstrated to be a good ionization method for analyzing these metabolites by mass spectrometry. The metabolites and their mode of conjugation found in the three different species studied are summarized in Table 3.

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