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CHROMATOGRAPHIC SEPARATION AND QUANTITATIVE DETERMINATION OF THE METABOLITES OF DI-(2-ETHYLHEXYL) PHTHALATE FROM URINE OF LABORATORY ANIMALS

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SUMMARY

Free, glycine-conjugated, and glucuronide-conjugated metabolites of di-(2-ethylhexyl) phthalate may be stripped from urine with XAD-2 resin, derivatized, and quantitatively analyzed by liquid chromatography on a nitrile column with UV and/or radioactivity monitors. One class of metabolites requires reversed-phase chromatography or gas–liquid chromatography for its resolution. Relative molar responses of the hydrogen flame-ionization detector to these metabolites have been determined. Packed gas chromatography columns (OV-3, OV-210, cyclohexanedimethanol succinate) and fused-silica capillary columns (SP2100 and FFAP) are useful for quantitative analysis under appropriate conditions. The simplest gas chromatographic procedure permitting complete quantitative analysis requires hydrolysis of conjugates, formation of methyl esters of carboxyl groups, butyration of hydroxyl groups and chromatography on OV-3. Typical distributions of di-(2-ethylhexyl) phthalate metabolites in urine from mice, hamsters and guinea pigs are presented.

INTRODUCTION

Di-(2-ethylhexyl) phthalate (DEHP) is one of the most widely used plasticizers, particularly for vinyl plastics, amounting to roughly one fourth of total plasticizer production[1]. Although the acute toxicity of this compound is very low (oral LD_{50} > 26 g/kg in rats[1]), the fact that everyone has some exposure to DEHP from vinyl plastics and that hemodialysis patients and those who must receive extensive therapy involving blood transfusions may receive considerable doses of DEHP leached from blood bags and medical tubing[2–4], have stimulated continuing interest in the metabolic fate and biological effects of this compound.

Phthalate plasticizers have been the subject of two fairly recent symposia to review their toxic potential[5,6]. The more recent of the two was held shortly after an announcement[7] by the National Toxicology Program (NTP) that DEHP had been found to be a hepatocarcinogen in both rats and mice when fed at levels of 0.6–1.2% of the diet for two years. Unfortunately, the NTP study had not been published by the time of the phthalate conference[6].

Publications from our laboratory[8-10] have suggested that the wide species differences in the metabolism of DEHP may have relevance to the potential toxicity of this compound. Biological activities such as reducing susceptibility to the toxicity of parathion[11], reducing levels of triglycerides and cholesterol in blood[12], or testicular atrophy[13] may also be associated with DEHP metabolites rather than the parent compound. Therefore the much more extensive oxidative metabolism of DEHP in rodents than in primates may be significant in health hazard assessment.

The present report describes a series of procedures we have found effective for the separation and analysis of the metabolites of DEHP that may be found in urine of laboratory animals. We have previously described thin-layer chromatographic[8], high-performance liquid chromatographic (HPLC)[14] and gas chromatographic (GC)[10] techniques applicable to the metabolites produced by rats. Since rats do not excrete conjugates of DEHP metabolites as do most other species[10], more versatile techniques were needed to extend studies to other common laboratory animals.

MATERIALS

HPLC was performed using a Spectra-Physics Model 8000B instrument equipped with a variable-wavelength UV monitor and a built-in Model 4000 data system for peak area integration. The same data system was used to process GC signals from a Varian Model 3740 chromatograph's hydrogen flame ionization detector. Fractions from HPLC were collected on a time basis with an Isco fraction collector and radioassayed by liquid scintillation counting. GC–mass spectrometry (MS) conditions have been described previously[15]. XAD-2 resin was from Supelco (Bellefonte, PA, U.S.A.).

All solvents used for extraction or chromatography were glass-distilled "pesticide grade", either Omnisolve or Burdick & Jackson. Reagent chemicals were used as received from Fisher Scientific, (Raleigh, NC, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.). The only chemical occasionally found contaminated with phthalates was anhydrous sodium sulfate. When necessary this was washed with methylene chloride prior to use. Di-(2-ethylhexyl)-[7-^{14}C]phthalate, 10.06 mCi/mmole, was synthesized as described previously[8], as were unlabeled DEHP and *mono*-2-ethylhexyl phthalate (MEHP). Other phthalate standards were obtained commercially. Glycine and taurine, labeled with ^{14}C, were obtained from ICN Radioisotope Division (Irvine, CA, U.S.A.) and used as received.

The enzymes aryl sulfatase (Type VI), carboxypeptidase A, acylase I, and β-glucuronidase (bovine liver) were from Sigma (St. Louis, MO, U.S.A.). Their activities were confirmed using the incubation conditions employed by Sigma. The animals used in this study were CD-strain rats (Charles River), Syrian Golden Hamsters, Hartley Albino Guinea Pigs, and CD-1 mice. Males were used throughout for comparison purposes, and all animals were approximately 4 months of age.

METHODS

(A) Treatment of animals

For routine metabolite identification, animals were administered, by stomach tube, two doses of DEHP at 24-h intervals. Urine was collected over a thymol crystal

in a glass beaker for a total of 48 h following the initial dose of DEHP, but removed from the metabolism cage and stored frozen at $-20°$ at 24-h intervals. In most cases both urine samples from a given animal were combined prior to analysis, except when stated otherwise in what follows. Animals were fed NIH 31 chow and water *ad lib.* and housed in standard metabolic cages to permit separate collection of urine and feces.

DEHP was given as a solution in cottonseed oil analyzed to ensure a peroxide value well below 0.04 (ref. 16) and absence of detectable aflatoxins. The total volumes administered (DEHP + cottonseed oil) were 0.1 ml for mice, 0.2 ml for rats, 0.15 ml for hamsters and 0.25 ml for guinea pigs. The maximum single dose of DEHP was 360 mg/kg body weight for mice, 180 mg/kg for rats and guinea pigs, and 20 mg/kg for hamsters. These limits were chosen arbitrarily on the basis of relative typical levels of DEHP-hydrolase activity in the intestines of these species[17]. When [7-^{14}C-]DEHP was given, it was first combined with unlabeled DEHP and cottonseed oil to give the desired specific activity. Dosages of ^{14}C did not exceed 10 μCi per animal.

Urine from these animals was analyzed for pH and residual β-glucuronidase activity (using phenolphthalein glucuronide), since these factors influence the stability of the metabolites. Typical values for urine pH were: rat 8, mouse 6, hamster 7 and guinea pig 9. Typical values for β-glucuronidase activity in nanomoles of phenolphthalein liberated per min per ml of urine at pH 4.5 were: rat 2.83, mouse 3.66, hamster and guinea pig none detectable.

(B) Synthesis of reference compounds

MEHP–Glycine methyl ester. MEHP (0.1 mmole) in 3 ml of tetrahydrofuran was stirred for 15 min at room temperature with 0.108 mmole of tri-*n*-butylamine and 0.1 mmole of isobutyl chloroformate. The resulting mixed anhydride was added to 0.2 mmole of glycine methyl ester hydrochloride in 2 ml of water + 0.2 ml of 1 *N* NaOH. After stirring for 10 min, 4 drops of pyridine were added and stirring continued another 15 min. The reaction products were diluted with 30 ml of *n*-hexane–ethyl acetate (1:1) and washed with 25 ml of 0.4 *M* aqueous K_2CO_3. The organic phase was washed with 25 ml portions of water, 0.5 *N* HCl, and water, then dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness in a rotary evaporator. The yield of desired product was 80% relative to MEHP.

Phthaloyl glycine methyl ester. Commercially available phthaloyl glycine (50 mg) was esterified by heating for 1 h at 100° in 1 ml of 14% BF_3 in methanol. The product was recovered by adding 10 ml of water and 5 ml of diethyl ether, washing the ether phase once with water, and evaporating the ether under nitrogen.

Phenylaceturic acid methyl ester. The N-hydroxysuccinimide ester of phenylacetic acid (1 mmole, Sigma), 1 mmole of glycine methyl ester hydrochloride and 1 mmole of redistilled triethylamine were stirred at room temperature in 25 ml of 1,2-dimethoxyethane for 1 h. After filtration through glass fiber filter paper, the filtrate was poured into 100 ml of ice cold water. The resulting suspension was extracted with 75 ml of diethyl ether, the ether phase was dried with anhydrous Na_2SO_4, and rotary evaporated. The residue was transferred to a small vial with methylene chloride and blown dry under nitrogen. The yield was only 95 mg.

Characterization of products. Each of the above products was at least 98% pure both by normal-phase HPLC (section *G* below) and by GC on OV-3 and OV-210

(section I). Infrared spectra of neat films on NaCl show disappearance of free carboxyl (peaks at 1690 and 900 cm^{-1}, broad hump around 3000 cm^{-1} in parent compounds before esterification), the presence of aliphatic ester (1749 cm^{-1}), and the presence of secondary amide structure (3325, 1660, 1520 cm^{-1}, bonded state) for MEHP–glycine methyl ester and phenylaceturic acid methyl ester. Phthaloylglycine methyl ester, being an imide, lacks the amide I and II bands but shows ν (C–N) at 1220 cm^{-1}. The expected molecular weights were confirmed by chemical ionization MS[15].

(C) Recovery and esterification of metabolites from urine

Although the DEHP metabolites in rat urine can be extracted with high recovery, the conjugated metabolites found in urine of animals other than rats are too water soluble for direct extraction. Instead we dilute the urine 1:2 with water, filter through glass wool, and add HCl to 0.1 *N*. XAD-2 resin is Soxhlet-extracted with methanol, slurry-packed into a 1 cm I.D. burette (plugged with glass wool) to a depth of 7 cm, and topped with a 2 cm layer of clean sand. After a prewash with 100 ml of water and 50 ml of 0.1 *N* HCl, up to 15 ml of diluted urine is run through. Elution with 170 ml of 0.1 *N* HCl followed by just enough hexane to push the remaining aqueous phase out of the column gives fraction A. Fraction B is eluted with 140 ml of methanol, and the combined organic eluate contains the DEHP metabolites.

The organic solvent is removed on a rotary evaporator. Small amounts of water carried into fraction B are eliminated by the addition of several small portions of methylene chloride to the residue and evaporating the azeotrope. The final residue is dissolved in 1 ml of methanol (any insoluble material is discarded) and esterified with diazomethane as described previously[8], being sure that enough diazomethane is used to esterify twice the number of milliequivalents of carboxyl as millimoles of DEHP given to the animal. This ensures a slight excess, since the color of the preparation will obscure the color of the diazomethane itself. Ethanol should not be used in the preparation of the diazomethane if it can be avoided. In general, the described procedure gives 94–96% recovery of total urinary ^{14}C when any of the species discussed in this paper are given [7-^{14}C]DEHP.

(D) Enzymatic hydrolysis

If the urinary metabolites are to be hydrolyzed with β-glucuronidase before analysis (*e.g.* ref. 10), then the dried residue from fraction B above is not treated with diazomethane. Instead, the residue is taken up in 5 ml of 0.2 *M* sodium acetate pH 5 containing 0.4 g of sodium sulfate. Bovine liver β-glucuronidase (Sigma type III) is added to give 15,000 Fishman units per ml and the preparation incubated at 37°C for 6 h. Add 0.8 ml of 6 *N* HCl, and extract four times with diethyl ether, 10 ml each time. The combined ether extract is dried with anhydrous sodium sulfate and concentrated with a rotary evaporator. Although the glucuronides of different DEHP metabolites are hydrolyzed at quite different rates, these conditions are sufficient for nearly quantitative hydrolysis of all the glucuronides so far encountered.

Attempts to cause hydrolysis with aryl sulfatase[18], carboxypeptidase A[19] or acylase I (which hydrolyzes glycine conjugates)[20] were without effect on the phthalate metabolites. We have thus far seen no evidence that DEHP metabolites are excreted as sulfate, glycine, or other amino acid conjugates by any of the species tested.

(E) Acylation reactions

Non-conjugated metabolites in the form of their methyl esters (a few mg) were treated with 1 ml of dichloroethane containing 0.5 *M* acetic anhydride or butyric anhydride, and 0.15 *M* perchloric acid at room temperature for 10 min[21]. Then 4 ml of dimethylformamide–pyridine–water (6:3:1) was added to hydrolyze the excess reagent. After 15 min the solution was diluted with 10 ml of water and the products extracted into 6 ml of diethyl ether. After washing twice with 5-ml portions of water the ether phase was dried with anhydrous Na_2SO_4, filtered, and the ether blown off with a stream of nitrogen. The residue was taken up in 1 ml of methylene chloride, washed with 2 ml of water, centrifuged, and blown dry. The acetates or butyrates were dissolved in methylene chloride for gas or liquid chromatography.

(F) Hydrolysis with sodium hydroxide

Total. If the urinary metabolites are either refluxed or held in a sealed tube at 100° for 90 minutes in 10 ml of 2 *N* NaOH or KOH, in 50% aqueous ethanol, all are hydrolyzed completely to *o*-phthalic acid[8]. If desired, suberic acid may be added as an internal standard for GC analysis of "total phthalate" content.

Conjugates–partial. Urine containing ^{14}C-labeled metabolites is diluted with three volumes of water. A 4-ml portion of diluted urine (equivalent to 1 ml of original urine) is treated with 0.44 ml of 1 *N* NaOH, then swirled gently at room temperature for exactly 10 min. Reaction is stopped by the addition of 1 ml of 6 *N* HCl, and the reaction mixture extracted 4 times with 10 ml of diethyl ether each time. The combined ether extract is dried with anhydrous sodium sulfate, the solvent is evaporated, and the residue esterified with diazomethane for analysis. This procedure will quantitatively hydrolyze the glucuronide esters, but not the much more stable glucuronide ethers[22]. Thus far all of the urinary glucuronides of DEHP metabolites appear to be esters.

(G) HPLC —normal phase

Standard conditions are as follows:

Column: 25 × 0.45 cm, Spherisorb S5CN (0.6 mmole cyanopropyldihydroxysilyl per gram) 5-μm particles, operated at 35°C with a constant flow-rate of 3 ml/min.

Solvent: non-linear gradient (Spectraphysics NL 2, concave) starting at 100% *n*-heptane (deaerated with helium) and programmed to *n*-heptane–tetrahydrofuran (50:50) (also deaerated with helium) in 30 min. The 50:50 mixture is held for 2 min, then switched to 100% tetrahydrofuran for 5 min to flush the column, and recycled to *n*-heptane for 15 min between runs.

Detector: UV monitor at 275 nm if variable or 280 nm if fixed. All of the metabolites have a molar absorbancy close to $1.2 \cdot 10^3$ at 275 nm, so peak areas should be close approximations of molar quantities.

Injection: samples are injected through a 10-μl sample loop, and pass through a 2-μm in-line filter before reaching the column. Since many of the more polar metabolites have very low solubility in heptane, we have found it effective to inject the samples in 10 μl of methylene chloride. All injected samples are in the form of methyl esters (*i.e.* have been treated with diazomethane before analysis).

(H) HPLC —reversed phase

Metabolites I–V are not resolved by normal-phase chromatography. When necessary to determine the distribution of radioactivity among these five compounds we collect them from the nitrile column above as a combined fraction, concentrate the solvent, and rerun the mixture on a 25 × 0.46 cm column packed with RP-8 (Spectra-Physics, 10-μm particle size) at 35°, 1 ml/min, with a solvent mixture of methanol–water (65:35). For a void volume of 2.23 ml, we observe the following capacity ratios (k') for the metabolites: I = 2.57, II = 2.89, III = 3.14, V = 3.35 and IV = 3.76. When the metabolites are not radiolabeled it may be more convenient to analyze the I–V pool by GC on OV-3 as described later.

(I) GC —recommended conditions

The metabolites of DEHP must be freed of the glucuronic acid moiety and esterified with diazomethane prior to GC. Retention indices, used to confirm the identifications of the metabolites, have been listed for a variety of liquid phases previously[10]. In general, we consider the pair of liquid phases OV-210 and OV-3 most powerful in confirming identifications. Retention indices are determined under isothermal conditions, however, and we have found that qualitative identification is best performed under GC conditions different from those yielding the most accurate quantitative measurements.

Analyses were performed using stainless-steel packed columns and fused-silica capillaries. In all cases the injection port was maintained at 250° and the hydrogen flame-ionization detector at 260°C. Splitless injection, purging after 15 sec, was used for the capillary column runs.

Routine analyses were made using a 3 m × 2 mm I.D. column packed with 3% OV-210 on 100–120 mesh Gas-Chrom Q. The column was linearly programmed from 150 to 195°C at 6°C/min, and operated at a helium flow-rate of 30 cc/min. Injections were 3 μl or less, with methylene chloride as solvent.

The fraction from HPLC containing metabolites I–V (see Table I) was analyzed using a 1 m × 2 mm I.D. column packed with 10% OV-3 on 100–120 mesh Gas-Chrom Z, at 180°C with a helium flow-rate of 40 ml/min. All five metabolites are well resolved. This column is also useful in that it effectively isolates metabolite X (Table I) for measurement without interference when the total metabolites are analyzed. However, metabolites III, VI, VII and IX all overlap on this column.

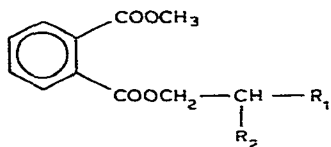
All of the metabolites are fully resolved except metabolites V and IX which overlap, when a 25 m × 0.23 mm I.D. capillary coated with free fatty acid phase (FFAP) is operated at 210°C with a helium head pressure of 24 p.s.i.g. On SP2100, 25 m × 0.15 mm capillary, with a helium head pressure of 20 p.s.i.g., held at 170°C for 12 min and then programmed to 200°C at 5°C/min, all metabolites except III and IX are resolved.

We also have use for a 2 m × 2 mm I.D. column packed with 3% cyclohexanedimethanol succinate on 100–120 mesh acid-washed Chromosorb W when metabolites from monkey or human urine are analyzed. This column is needed to resolve metabolites VII and VIII (Table I) at 200°C with a helium flow-rate of 33 ml/min.

The only system we have been able to find that will resolve all of the metabolites on a single packed column, is to form the butyrates after treatment with diazomethane, and chromatograph them on the OV-3 column described above. A linear

TABLE I

STRUCTURES OF DEHP METABOLITES AFTER TREATMENT WITH DIAZOMETHANE



Metabolite	R_1	R_2
I	$-CH_2-CO_2-CH_3$	$-CH_2-CH_3$
II	$-(CH_2)_3-CH_3$	$-CO_2-CH_3$
III	$-(CH_2)_2-CO_2-CH_3$	$-CH_2-CH_3$
IV	$-(CH_2)_3-CH_3$	$-CH_2-CO_2-CH_3$
V	$-(CH_2)_3-CO_2-CH_3$	$-CH_2-CH_3$
VI	$-(CH_2)_2-CO-CH_3$	$-CH_2-CH_3$
VII	$-(CH_2)_3-CH_3$	$-CH_2-CH_2OH$
VIII*	$-CH_2-CHOH-CH_2CH_3$	$-CH_2-CH_3$
IX	$-(CH_2)_2-CHOH-CH_3$	$-CH_2-CH_3$
X	$-(CH_2)_3-CH_2OH$	$-CH_2-CH_3$
XI**	$-(CH_2)_3-CH_3$	$-CH_2-CH_3$

* Absent in the rat.

** Mono-(2-ethylhexyl) phthalate.

program from 160–200°C at 6°C/min gives complete separation. OV-210 can also be used, but DEHP itself may interfere with quantitation of metabolite VI.

RESULTS AND DISCUSSION

An HPLC chromatogram showing DEHP metabolite standards originally derived from rat urine is shown in Fig. 1. The UV monitor response is shown. Fig. 2, compressed by using a slower recorder chart speed, shows the pattern of metabolites (UV monitor) seen in urine from CD-1 mice. Fig. 2 shows numerous peaks eluting later than those in Fig. 1 and which were found to be conjugates. No components were detected in urine from hamsters or guinea pigs that were not also present in rat or mouse urine.

As mentioned earlier, neither aryl sulfatase, acylase I nor carboxypeptidase A had any significant effect on the DEHP metabolites. Although oral administration of ^{14}C-labeled taurine to mice resulted in excretion of radioactivity in the urine, none of the radioactivity became organic soluble under the workup conditions used for phthalate metabolites. Administration of ^{14}C-labeled glycine did indeed lead to excretion of labeled compounds that passed through the workup and appeared as radioactive peaks under these HPLC conditions; however, none of them co-chromatographed with phthalate metabolites. The major [^{14}C]glycine-derived urine constituents co-chromatographed with the methyl esters of glycine, hippuric acid, and phenylaceturic acid. The latter compounds did not become labeled when mice were given [^{14}C]DEHP. It was shown, however, that if glycine conjugates of DEHP metab-

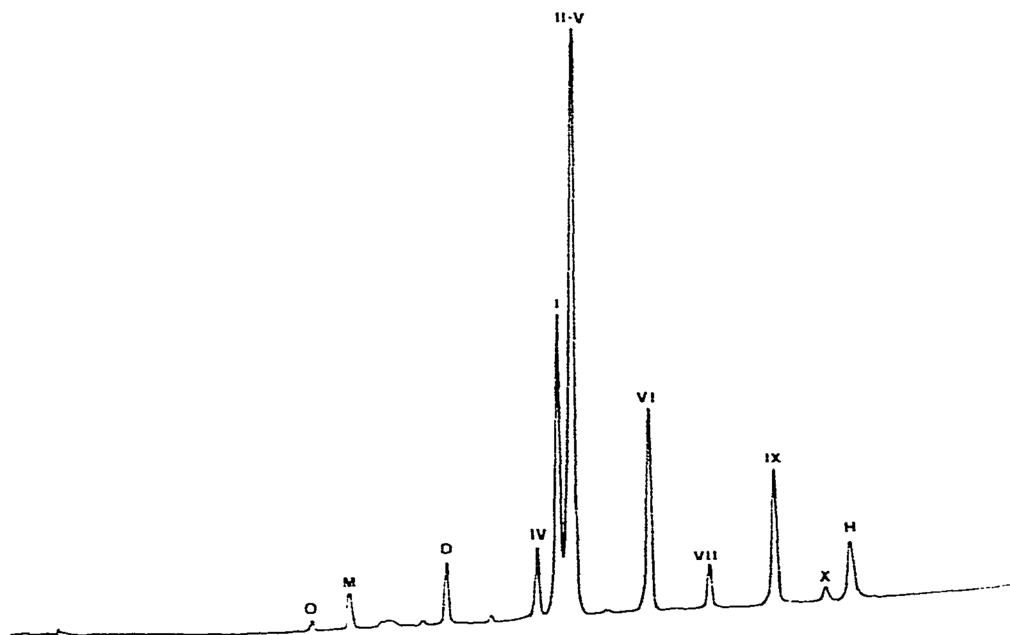


Fig. 1. HPLC on Spherisorb S5CN (Nitrile Phase) of rat urinary metabolites of DEHP after esterification with diazomethane. Chart speed 0.5 cm min before photographic reduction. 0 = DEHP (added as marker). other identifications as in Fig. 2.

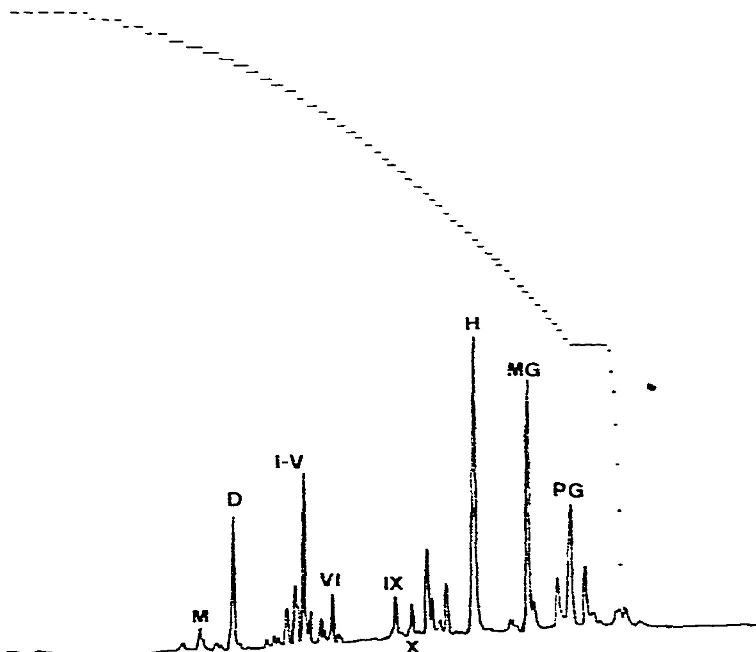


Fig. 2. HPLC on nitrile phase of mouse urinary metabolites of DEHP as methyl esters. Chart speed 0.25 cm/min before photographic reduction. M = MEHP; D = dimethyl phthalate; I–X = metabolites identified in Table I; MG = MEHP-glucuronide; PG = glucuronides of metabolites more polar than MEHP, H = methyl hippurate (not a phthalate metabolite). UV monitor output.

olites should be produced in some as yet untested species, they would chromatograph on this nitrile phase between the free metabolites and the glucuronide conjugates. The synthetic glycine conjugate of MEHP, for example, elutes immediately after metabolite X.

Fig. 3 shows a comparison of the elution patterns seen when one third of a sample of mouse urine containing metabolites derived from [7-^{14}C]DEHP was run without hydrolysis, one third was first partially hydrolyzed with β-glucuronidase (for only 3 h instead of 6), and the last third was hydrolyzed with dilute NaOH for 10 min. This figure is based on monitoring for radioactivity and appears to show that the major late-eluting metabolites are glucuronide esters. To confirm this, the major component eluting between fractions 55 and 57 was collected and pooled from five HPLC runs. Half of the recovered material was hydrolyzed with dilute NaOH as described above, re-treated with diazomethane, and re-chromatographed on the nitrile phase HPLC column. The only detectable radioactive product co-chromato-

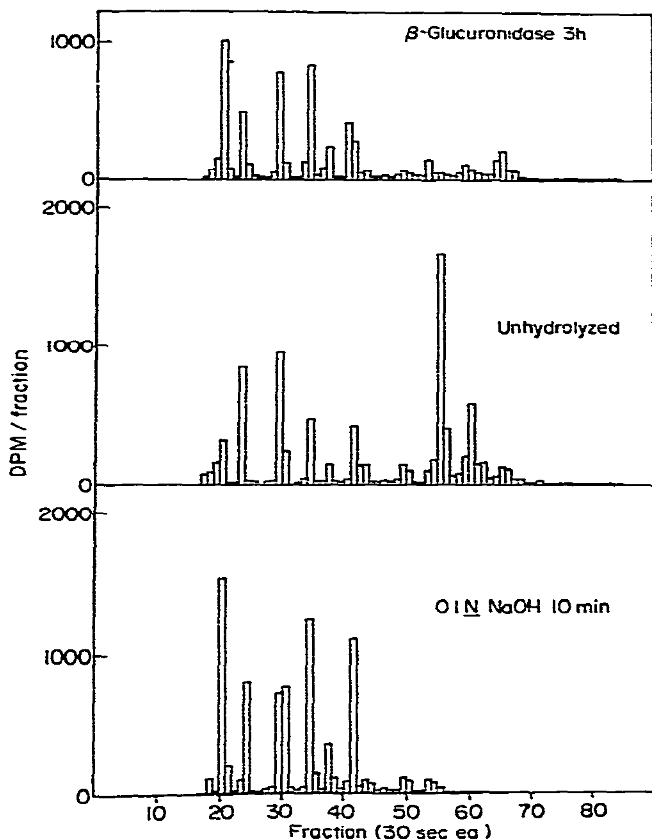


Fig. 3. HPLC on nitrile phase with radioactivity monitoring. Metabolites of ^{14}C-labeled DEHP were recovered from mouse urine, and esterified with diazomethane either with no hydrolysis (center), after partial hydrolysis with β-glucuronidase (top), or after hydrolysis of ester type glucuronides with 0.1 N NaOH at room temperature (bottom). MEHP peaks in fraction 21, dimethyl phthalate in 24, I–V in 30–31, VI in 34, VII in 38, IX in 42 and X in 44.

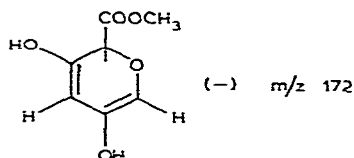
TABLE II

CHEMICAL IONIZATION MASS SPECTRA OF POOLED FRACTION 53–58, NITRILE HPLC, OF CH_2N_2-TREATED MOUSE URINARY METABOLITES OF DEHP

Positive-ion spectrum		*Negative-ion spectrum*	
m/z	*Rel. abundance*	*m/z*	*Rel. abundance*
55	27.7	71	4.8
57	39.3	86	1.6
69	16.5	99	9.7
71	43.0	113	4.3
73	10.6	117	8.1
85	18.7	129	2.7
97	14.9	147	17.6
111	5.4	148	93.3
113	26.2	149	5.8
115	9.4	157	2.9
149	100	158	9.1
150	6.7	164	14.9
167	19.4	165	100
173	5.6	166	7.0
277	1.4	171	6.6
279	16.7	172	32.1
		277	55.7
		278	11.4
		468	5.9
		469	0.2

graphed with methyl MEHP. The other half of the purified material was examined by methane-supported positive-ion chemical ionization MS and also by methane-oxygen negative-ion chemical ionization MS as described previously[15]. Samples were introduced by direct probe. The resulting spectra are summarized in Table II.

The base peak in the negative ion spectrum is at *m/z* 165 and the second most abundant peak at *m/z* 148. These are assigned to the monovalent anion of phthalic acid and to phthalic anhydride respectively. The major fragment at *m/z* 277 corresponds to the anion of MEHP itself, and this is confirmed by the presence of a significant fragment at *m/z* 113 in the positive ion spectrum (assigned to the ethylhexyl moiety). The molecular weight appears to be 468. The fragment at *m/z* 172 is thought to be analogous to the hydroxymethylfurfural dehydration product of hexoses, *i.e.*



and may serve as a diagnostic fragment for detection of methyl esters of glucuronides. We are investigating this possibility further at the present time. In general, the spectra are consistent with the identification of this metabolite as the methyl ester of the

glucuronide ester of MEHP. Although we have not obtained the same degree of characterization for the other late-eluting metabolites, their hydrolysis by β-glucuronidase and dilute NaOH accompanied by corresponding increases in metabolites I–X suggests by analogy that all of the DEHP metabolites are present in mouse urine as a mixture of free and glucuronide conjugated forms.

The minor radioactive components eluting between metabolite X and MEHP-glucuronide in Fig. 3 have been only partially characterized. Saponification of the urine or of just these minor products isolated together by prep. HPLC yields phthalic acid as the only radioactive product. These components are not hydrolyzed by enzymes or dilute NaOH. Following treatment of the previously isolated components with acetic anhydride, they chromatograph in the region of metabolites V and VI, suggesting the presence of only ester groups after acetylation. At present we believe that these are products of the oxidation of both branches of the ethylhexyl side chain. For example, if the ethyl group of metabolite I were oxidized to $-CH_2CH_2OH$, it would have chromatographic properties like these minor metabolites. The small amount of material available has so far prevented our identifying these products with certainty. Each of the components identified as metabolites I–X in the figures has however, been isolated by preparative HPLC and its identity confirmed by GC–MS[15].

In order to perform quantitative GC, it was necessary to determine relative molar responses of the hydrogen flame ionization detector for these metabolites. To accomplish this, a rat was given a 100 mg, 5 μCi, dose of [7-^{14}C]DEHP in cottonseed oil and the 24-h urine processed for the preparation of methyl esters as described

TABLE III

RELATIVE MOLAR FLAME DETECTOR RESPONSES FOR THE METABOLITES OF DEHP AS METHYL ESTERS

These values apply when peak areas are measured by electronic integrator. DMP = dimethyl phthalate; M,EHP = methyl, 2-ethylhexyl phthalate; DEHP = di-(2-ethylhexyl)phthalate; I–X as identified in Table I.

Metabolite	*Relative molar response, DEHP = 1.000*
DMP	0.329
M,EHP	0.745
DEHP	1.000
I	0.580
II	0.77
III	0.60
IV	0.51
V	0.675
VI	0.607
VII	0.37
VII-butyrate	0.58
IX	0.54
IX-butyrate	0.65
X	0.33
X-butyrate	0.80

above. The molar distribution of metabolites was determined from the distribution of radioactivity on the nitrile-phase HPLC column, separating the fraction corresponding to metabolites I–V on the C_8 reversed-phase HPLC column. Results from three runs were averaged. The remaining sample was then split, half being analyzed by GC directly and half being treated with butyric anhydride before GC. Components were quantitated relative to an added internal standard of DEHP itself, since DEHP was found to comprise less than 0.05% of the ^{14}C excreted in the urine. Each metabolite was quantitated from peak area measurements on whichever GC column best resolved it from other urinary constituents, and the final estimation was the mean of 4 determinations. The relative molar responses are summarized in Table III, and are considered to be most accurate for the more abundant metabolites.

Since we have previously found that peak area percentages from the flame detector correlated quite well with peak area percentages from the sum of the m/z 163 + 181 ion fragments of electron impact GC–MS (correlation coefficient, $r = 0.992$, n

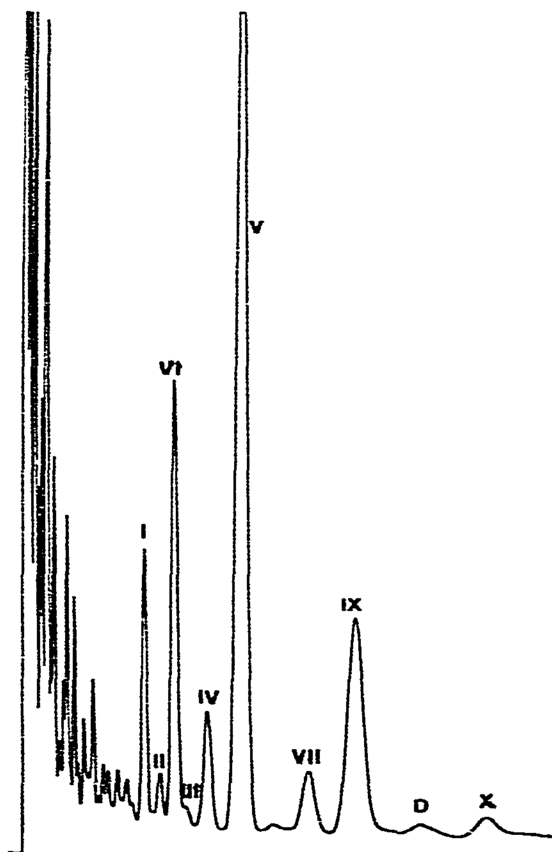


Fig. 4. GC of butyrates of methyl esters of rat urinary metabolites of DEHP on OV-3. A 1 m × 2 mm I.D. column packed with 10% OV-3 on 100–120 mesh Gas-Chrom Z was temperature programmed from 160–200°C at 6°C/min with a helium flow-rate of 43 ml/min. D = DEHP, other metabolites as designated in Table I.

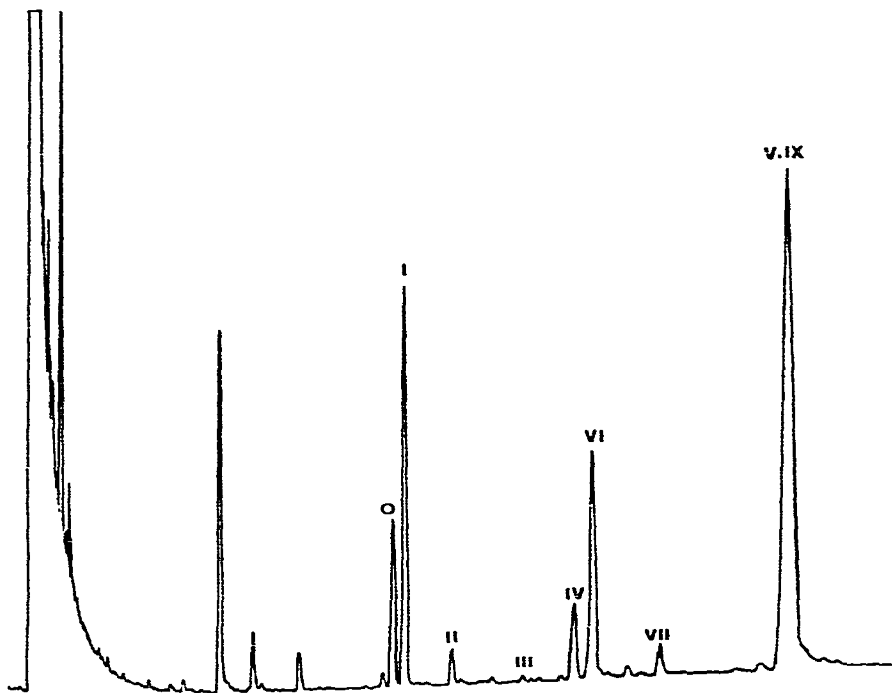


Fig. 5. Capillary column GC of rat urinary metabolites on FFAP. A 25 m × 0.23 mm, fused-silica capillary, coated with FFAP, was operated isothermally at 210° with a helium pressure of 24 p.s.i.g.

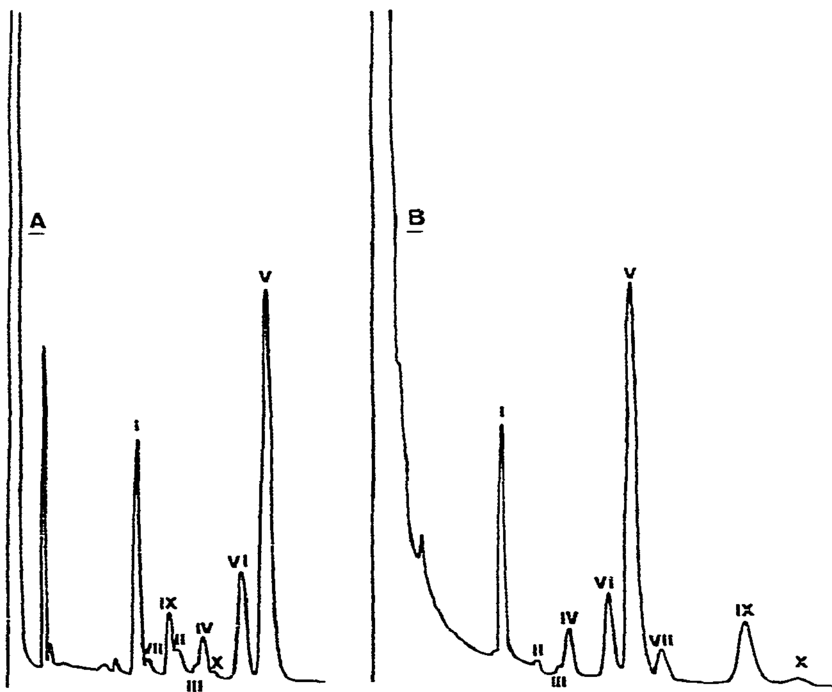


Fig. 6. GC of rat urinary metabolites of DEHP on OV-210 at 195°. A = Free methyl esters, B = butyrated methyl esters. For details see Methods section of text.

= 8 samples), the relative molar responses in Table III may also be applied to quantitative analysis by GC–MS. Similarly, the UV monitor peak areas correlated well with the ^{14}C distributions ($r = 0.990$, $n = 8$), indicating that no correction factors are necessary for the UV monitor.

Separation of the butyrates is shown in Fig. 4; all of the metabolites from rat urine can be seen. A representative chromatogram of the rat urinary metabolites on an FFAP capillary column is shown in Fig. 5. Metabolite V amounts to approximately 50% of the DEHP metabolites in rat urine, so its peak shows the effects of overloading in Fig. 5. Fig. 6 shows a typical gas chromatogram on OV-210. Several components overlap, but this column has advantages for routine use in GC–MS.

Several factors can invalidate GC analysis of these metabolites unless suitable precautions are taken. Metabolite IX, the secondary alcohol derivative, can autoxidize to metabolite VI (ketone), and is particularly prone to do so if the corresponding HPLC fraction, containing tetrahydrofuran, is evaporated to dryness. The butyrate of IX is stable, however. Metabolites VII and IX have a tendency to dehydrate and metabolite II to decarboxylate, if the solution in which they are injected into a gas chromatograph is at all acidic. Dimethyl phthalate, formed from free phthalic acid by diazomethane, is much more volatile than the other metabolites and can be lost if heat is used while evaporating solvent from its solutions.

Table IV summarizes "typical" distributions of DEHP metabolites in urine from CD-1 mice, Syrian Golden hamsters and Hartley Albino guinea pigs, based on distributions of ^{14}C from orally administered [7-^{14}C]DEHP. These results are presented only as illustrations, since dosing levels were not the same in the various species. In general, we do not find conspicuous effects of dose, age of animal, etc. on the metabolite distributions; nonetheless, the comparative data in Table IV should

TABLE IV

TYPICAL DISTRIBUTIONS OF METABOLITES IN URINE OF MOUSE, HAMSTER AND GUINEA PIG

Number in parentheses is percentage of that metabolite found in the form of glucuronide ester conjugate. Designations as listed in Table I.

Metabolite	*Molar percentage in 48-h urine of:*		
	Mouse	*Hamster*	*Guinea pig*
DEHP	0.5 (0)	0.3 (0)	–
DMP	12.4 (29)	9.5 (14)	5.4 (55)
M.EHP	18.6 (79)	4.5 (39)	71.2 (74)
I	16.8	13.0	2.4
II	1.0	0.1	0.4
III	0.4 (45)	0.3 (20)	0.5 (16)
IV	0.8	0.4	0.4
V	1.1	14.0	6.9
VI	14.9 (81)	10.2 (6)	1.1 (0)
VII	7.2 (77)	4.9	0.8 (23)
IX	12.3 (70)	32.7 (11)	3.4 (50)
X	2.2 (63)	1.9	1.3 (72)
Uncertain	11.5 –	6.1	6.2

not be carried too far. One interesting observation was that guinea pigs appear to perform almost no oxidation of DEHP, which is consistent with their known metabolic limitations in mixed-function oxidase activity.

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