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Analysis of Di-n-butylphthalate Biotransformation in Cattle by Liquid Chromatography/Ion Trap Mass Spectrometry/Mass Spectrometry

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The nature of products of contamination intake were investigated in cattle dosed with [^{14}C]di-n-butylphthalate (DBP). Radio-labelled metabolites were extracted from bile, faeces, plasma and urine onto solid-phase media, fractionated by ion-exchange chromatography, separated by reverse phase HPLC and analysed by negative ion atmospheric pressure chemical ionization mass spectrometry^n (LCQ, Finnigan). All matrices contained a common major metabolite [deprotonated molecular ion $(M\text{-}H)^-$ m/z 221] which coeluted with and had an identical daughter ion spectrum to reference monobutylphthalate (MBP). MBP was metabolised to a β-glucuronidase sensitive compound $(M\text{-}H)^-$ m/z 397 whose spectrum contained daughter ions (m/z 175 and 221) consistent with the parent glucuronide. A further three β-glucuronidase resistant radio-labelled metabolites were also produced ($M\text{-}H^-$ m/z 165, 193 and 237); comparison of daughter ion spectra with those of reference MBP and phthalic acid indicated identity with phthalic acid, monoethylphthalate (MEP) and monohydroxybutylphthalate (MHBP) respectively. The presence of a benzoate daughter ion (m/z 121) in all spectra was indicative of side chain biotransformation. Both MBP and MEP contained a phthalate daughter ion (m/z 165) indicating loss of a butyl and ethyl side chain respectively. A daughter ion of m/z 89 derived from the side chain provided evidence that the third metabolite was MHBP. Incubation of DBP with isolated bovine hepatocytes produced the same metabolites and provided relatively clean samples for LC/MS^n analysis. Detection of these DBP metabolites in meat or dairy food products will provide evidence for environmental exposure and biotransformation *in vivo*, whereas the presence of the parent compound would suggest contamination during food processing and packaging.

KEYWORDS: radio-HPLC-MS^n; biotransformation; dibutylphthalate; bovine; food contamination

INTRODUCTION

Phthalic acid esters (PAEs) are produced in large quantities by industry and they are used world-wide in many applications, for instance as plasticizers, fixatives, detergents, lubricating oils and solvents.[1] Unrestricted use of such products, the propensity of PAEs to leach from plastics[2] and their chemical stability has produced widespread environmental contamination. Although the acute toxicity of PAEs in laboratory animals is low (8 g/kg),[3] chronic exposure studies indicate that lower doses may reduce the fertility of male rats[4] and certain phthalates have been associated with weak endocrine disrupting activity.[5] PAEs are fat soluble and have been found, albeit at low levels, in many foods,[6,7] including infant milk formulae.[8] These findings have increased concerns regarding the risk that PAEs may present to the consumer.[9] Contamination of food may occur through at least two distinct routes; lipophilic environmental pollutants are known to accumulate in animal tissues, and several reports[10–12] have described the migration of plasticizing agents into foods during processing and following packaging. Clearly, whatever the route of food contamination with PAEs, there is a need to trace sources and reduce levels of food contamination.[13]

PAEs are readily biotransformed in laboratory animals to a range of water soluble metabolites and excreted in urine.[14] In all species studied, the primary step of PAE biotransformation is hydrolysis to a monophthalate ester.[15] The remaining alkyl side chain of the monophthalate ester may be further metabolised by ω and ω-1 oxidation to a range of polar metabolites including alcohols, aldehydes, ketones and carboxylic acids. Further biotransformation involves β-oxidation of this alkyl side chain with the loss of a two carbon fragment.[16,17] Such metabolic activity provides a means of distinguishing between routes of food contamination; detection of unchanged parent compound would suggest contamination during processing and storage whereas metabolites would be more indicative of biotransformation *in vivo*. GC-MS has been widely used to detect and identify metabolites of PAEs,[18–20] although this approach is not well suited to the detection of water soluble radio-labelled metabolites which require prior derivatisation. LC/MS^n offers several distinct advantages for such metabolic studies since this enables liquid

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chromatography for metabolite separation with simultaneous radio detection and structural analysis by MS^n. Further, the ability to perform MS^n experiments on the molecular ions of metabolites enables acquisition of structural information for metabolite identification without either mass spectral contamination from coeluting extractives or recourse to derivatisation for GC-MS analysis. The aim of the present study was to determine the metabolic fate of di-n-butylphthalate in the bovine using liquid chromatography with bench-top ion trap mass spectrometry^n for the identification of radiolabelled metabolites.

EXPERIMENTAL

Chemicals

DBP, phthalic acid (both of AR grade) and *Helix pomatia* β-glucuronidase (also contains sulphatase activity) were obtained from Sigma, Poole, Dorset, U.K. HPLC grade solvents were from Rathburn Chemicals, Walkerburn, Scotland. Monobutylphthalate was supplied by ChemService, West Chester, PA. USA. Di-n-butyl[carboxyl-^{14}C]phthalate, 962 MBq/mmol, was custom synthesised by Amersham International (Little Chalfont, Bucks, U.K.) to a purity of 99.5%.

Animals

Two female Friesian cross heifers (126 and 142 kg) were surgically prepared to provide gall-bladder cannulae externalised for collection of bile and re-entrant to the duodenum.[21] After recovery, animals were housed in metabolism cages designed for the collection and separation of urine and faeces, and administered, by intra-ruminal injection, [^{14}C]DBP (185 MBq; diluted in ethanol (10 ml) with unlabelled compound) at a total dose of 10 g per animal. Blood (jugular venepuncture), bile and representative samples of urine and faeces were taken at appropriate intervals (1, 2, 4, 6, 9, 12, 14, 18, 20, 24, 28, 30, 36, 48, 54, 72, 78 and 96 hours) after dosing.

Bovine hepatocytes

Isolated hepatocytes were prepared from the caudal lobe of bovine liver as described previously[22] to an initial viability of 95% and incubated in suspension culture (1×10^6 cells/ml) with 10 μM DBP containing 1.7 kBq[^{14}C]DBP/ml. Culture medium was sampled at intervals, for up to 20 hours and separated from hepatocytes by centrifugation at 2000 × *g* and stored at −20 °C.

Metabolite extraction

Bile, urine, plasma and faeces sampled approximately 20 hours after dosing were selected for extraction with C18 media and further fractionated on the basis of functional groups by cation and anion exchange chromatography[23] since these samples contained the highest concentrations of radioactivity. Biotransformation of DBP was complete after 20 hours incubation with bovine hepatocytes and these incubates were extracted and fractionated in a similar manner.

C18 extraction. Urine and hepatocyte medium (2 ml) were diluted with an equal volume of 0.02% formic acid and applied to prewashed [methanol followed by 0.02% formic acid (10 ml)] C18 (MegaBond Elut, Varian, Harbor City, CA, USA) cartridges at ambient temperature. Cartridges were washed with 0.02% formic acid (3 × 10 ml) and bound radiolabelled material eluted with methanol (5 ml). Faeces (4 g) were homogenized in water (2 × 10 ml), centrifuged at 1400 × g and the aqueous supernatants were extracted as above. Plasma samples were loaded on to C18 columns at 64 °C to reduce protein binding and improve the recovery of radioactivity.[24] Methanol eluates were taken to dryness at 45 °C under nitrogen and either reconstituted in the mobile phase for radio-HPLC analysis or further group fractionated by cation and anion exchange chromatography.

Cation-exchange. C18 extracts were dissolved in 70% aqueous methanol (5 ml) and applied to SP-Sephadex columns (20 × 15 mm, in the hydrogen form) and rinsed with 70% aq. methanol (5 ml) followed by 0.3 M ammonia in 70% aq. methanol (2 × 5 ml).

Anion-exchange. Cation exchange eluates from the methanol fraction were taken to dryness, dissolved in 70% aq. ethanol (10 ml), applied to DEAP-Lipidex columns (20 × 15 mm, in the acetate form) and sequentially eluted with a series of displacers of increasing ionic strength consisting of 70% aq. ethanol (15, then 5 ml), 0.05 M acetic acid in 70% aq. ethanol (1 × 5 ml), 0.25 M formic acid in 70% aq. ethanol (4 × 5 ml), 0.3 M acetic acid 0.3 M potassium acetate in 70% aq. methanol (4 × 5 ml) and finally 0.3 M acetic acid potassium hydroxide in 70% aq. methanol (4 × 5 ml). Fractions containing radioactivity were taken to dryness and dissolved in the mobile phase for analysis by radio-HPLC-MS^n. Recovery of radioactivity during extraction procedures was continuously monitored by counting aliquots of each fraction and starting matrix (10–500 μl) by liquid scintillation counting.

β-Glucuronidase/sulphatase hydrolysis of metabolites. C18 extracts of bile and hepatocyte culture medium were dissolved in 50 mM sodium acetate buffer (pH 4.5, containing 100 mM NaCl) and incubated for 20 hours at 37 °C, with or without *Helix pomatia* β-glucuronidase/aryl sulphatase (1.4 mg ml^{-1}), prior to analysis by radio-HPLC-MS^n.

HPLC. Metabolites of [^{14}C]DBP in extracts of bile, urine, plasma and hepatocyte culture medium were separated by reversed phase radio-HPLC, using a Waters Spherisorb S5 ODS1 4.6 × 250 mm column and detected with an A500 radiodetector (Packard) with the MS in series. Metabolites were chromatographed at a flow rate of 1.0 ml min^{-1} using a linear gradient mobile phase ranging from 20% formic acid (0.02% v/v): 80% acetonitrile to 100% acetonitrile over 15 minutes and maintained at 100% acetonitrile for a further 5 minutes.

Under these chromatographic conditions reference solutions of phthalic acid, MBP and DBP eluted from the column with retention times of 7.0, 11.2 and 16.3 minutes respectively. The abundance of metabolites in radio-HPLC chromatograms were determined as a percentage of the total of all peaks with an area greater than 100 DPM.

MS^n analysis. Metabolites of DBP in HPLC effluent were tentatively identified by negative-ion atmospheric pressure chemical ionization mass spectrometry (LCQ, Finnigan, Hemel Hempstead, Herts) with a twin event, single segment experimental method. The primary event collected spectra over the mass range *m/z* 100–450 a.m.u. while the second performed data dependent MS/MS scanning on deprotonated pseudomolecular ions of potential DBP metabolites[14,17] in the event that their abundance exceeded an ion threshold setting of 10^4 counts; Pre-selected M-H$^-$ ions for data dependent MS/MS analysis included; *m/z* 165 (phthalic acid), 221 (monobutylphthalic acid), 235 (monobutanone phthalic acid), 237 (mono-n-hydroxybutylphthalic acid), 251 (mono-1-hydroxybutan-2-one phthalic acid and butanoic phthalic acid), and 397, 411, 413 and 427 (glucuronide conjugates of these metabolites). Other deprotonated pseudomolecular ions of DBP metabolites were selected for MS/MS analysis by matching coeluting peaks from selected ion and radio-HPLC chromatograms. A collision induced dissociation (CID) energy of 26% was selected for MS^n experiments by infusion of a 50 μM solution of MBP at 10 μl min^{-1} into the HPLC effluent and optimisation on an MBP daughter ion at *m/z* 177 (parent *m/z* 211). The heated capillary was set to 200 °C except for MS/MS detection of phthalic acid, where 150 °C improved sensitivity 50 fold. Ionization of the HPLC eluate was performed under the following settings; vaporizer temp 450 °C, source voltage and current 8 kV and 5 μA respectively, sheath gas and auxiliary gas flow rates (arbitrary units) 80 and 7 respectively and a capillary voltage of −4.00 V.

Identification of all metabolites was established on the basis of susceptibility to hydrolysis by treatment with β-glucuronidase/aryl sulphatase and daughter ion (MS^n) mass spectra where appropriate.

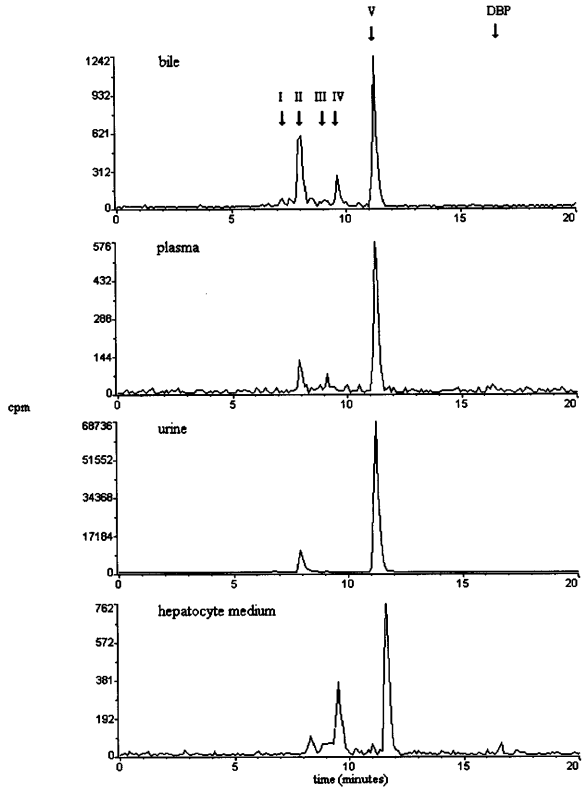

Figure 1. Representative radio-HPLC chromatograms illustrating the profiles of metabolites found in bile, plasma, urine and hepatocyte medium. Counts per minute (cpm) are plotted against time in minutes. Arrows above radio-peaks in the bile radio-HPLC chromatogram indicate retention time of metabolites I–V and DBP.

RESULTS

Metabolite extraction

Recovery of radioactivity from bile, faeces, plasma, urine and hepatocyte medium after extraction with C18 cartridges was (mean ± 1 s.d.) 93.5 ± 4.4, 89.6 ± 1.6, 76.2 ± 2.3 and 91.2 ± 1.1% respectively. Radioactive residues in these extracts were not subsequently retained by the SP-Sephadex cation exchange resin indicating an absence of metabolites containing an amino group. Essentially all radioactivity was absorbed on the

Table 1. Approximate retention times and abundances of metabolites I–V found in radio-HPLC traces of DEAP-Lipidex extracts of bile, plasma, urine, faeces (C18 extract) and hepatocyte medium[a]

Metabolite	Approximate retention time (min)	Relative abundance (%) Bile	Plasma	Urine	Faeces	Hepatocyte medium
I	7.0	2.8	—	0.4	—	—
II	8.0	33.2	14.8	12.7	48.5	6.0
III	9.0	3.7	7.5	0.4	—	6.0
IV	9.6	10.3	7.5	0.3	—	28
V	11.2	47.5	69.4	85.7	45.9	60
DBP	16.3	—	3.8	—	5.6	

[a] The abundance of each radio-labelled metabolite was determined as a percentage of the total peaks. Metabolites were assigned a Roman numeral I–V on the basis of the order of elution. Dashes indicate metabolite not found in the matrix.

DEAP-Lipidex columns and displaced by 0.25 M formic acid with an average recovery of $78 \pm 2.1\%$. Radio-labelled metabolites eluted by this displacer are associated with acidic metabolites.[24]

Radio-HPLC

Radio-HPLC profiles of metabolites present in DEAP-Lipidex extracts of bile, plasma, urine and hepatocyte medium are shown in Fig. 1, and were similar to those found in the cruder C18 solid phase extracts of these matrices. Faecal C18 solid phase extracts contained two metabolites (radio-chromatogram not shown), in equal proportions, which were readily identified by LC-MS^2 without recourse to further purification by ion exchange chromatography. All matrices contained a major metabolite which eluted at a retention time of approximately 11.2 minutes and a variety of minor metabolites, some specific to the matrix. The retention time of metabolites present in hepatocyte culture media extracts was slightly longer than that for other matrices. This may reflect either the absence of abundant coextractives or the presence of HEPES buffer derived from the cell culture media. The proportions of metabolites present in the 0.25 M formic acid fraction of Lipidex-DEAP column eluates from extracts of bile, plasma, urine and hepatocyte culture media and C18 extracts of faeces are summarised in Table 1. These metabolites are assigned Roman numerals I–V (Table 1) corresponding to the order of elution from the HPLC column.

Spectral analysis

Representative CID MS^2 spectra (A–E) corresponding to DBP metabolites (I–V) present in the 0.25 M formic acid fraction of Lipidex-DEAP column eluates are shown in Fig. 2.

Spectra A and E were obtained from parent ions of m/z 165 and 221 which corresponded to metabolites I and V and were consistent with the MS/MS spectra obtained with reference solutions of phthalic acid and MBP respectively. Mass spectral analysis of metabolite V in bile extracts was problematic due to substantial ion suppression in the selected (m/z 221) and total ion chromatogram at 11.2 minutes due to a coeluting extractive (m/z 465). This problem was partially overcome by decreasing the sample size and the maximum inject time of the ion trap.

Spectrum B was obtained from a parent ion of m/z 237, which corresponded to metabolite II. Comparison with spectrum E of MBP enabled deduction of the putative structure of metabolite II. Daughter ions of m/z 89 and 71 present in spectra B and E correspond to n-hydroxybutyl and butyl side chains derived from MHBP and MBP respectively. Corroboration that the site of hydroxylation of metabolite II is in the butyl chain was derived from the presence of unchanged benzoate fragment (m/z 121) in the spectra of both B and E. Determination of the precise site of hydroxylation of HMBP by mass spectral analysis was not possible since side chain fragments of MBP could not be produced by different CID energies (14–35%) and the hydroxybutyl fragment (m/z 89) was resistant to analysis by MS^3.

Spectrum C was obtained from a parent ion of m/z 397 which coeluted with β-glucuronidase sensitive metabolite III. The presence of a base ion at m/z 175, a fragment thereof at m/z 113 and an aglycone ion at m/z 221 is consistent with a glucuronic acid conjugate of MBP. Further analysis by MS^3 of the putative aglycone daughter ion (m/z 221) revealed a grand-daughter spectrum consistent with MBP.

Spectrum D was obtained from a parent ion of m/z 193, which corresponded to metabolite IV. Similarities between spectra D and E (MBP) provided sufficient structural information to enable the identification of IV as monoethylphthalate. Spectra D and E both contain daughter ions of m/z 121 and less intense ions of m/z 165 corresponding to benzoate and phthalate fragments respectively. Daughter ions (m/z 41 and 43) originating from the ethyl chain of MEP were not detected since they are below the MS^2 scan range of the LCQ. However, daughters at m/z 149 in the spectra of D and E are consistent with the loss of butoxy and ethoxy groups from monobutyl (E) and monoethyl phthalate deprotonated molecular ions respectively.

DISCUSSION

Radio-HPLC-MS^n proved an effective analytical approach for the detection and analysis of all DBP metabolites of significant abundance present in bovine bile, faeces, plasma and urine. In most cases this was achieved through data dependent MS^2 scanning for metabolites which have been identified in other species. Bile proved a difficult matrix for analysis since substantial ion suppression was evident at the anticipated elution time (11.2 mins) of MBP. Fractionation of bile by ion exchange chromatography failed to resolve this problem suggesting that the interfering materials may share functional groups with MBP. Candidate coextractives include bile acids which also contain a carboxyl group. Further, these problems were not encountered during the analysis of faecal extracts which may reflect reabsorption of bile acids by the gut. Similar profiles of DBP metabolites, with MBP predominant, were found in all matrices except faeces which contained equal proportions of MBP and MHBP. This may indicate either greater reabsorption of MBP or biotransformation of MBP by gut micro-flora to MHBP.

Comparison of daughter ion spectra with reference MBP and phthalic acid preparations enabled deduction of the structures of the metabolites I–V. The structure of these daughter ions, some common to several metabolites, are presented in Fig. 3. All unconjugated metabolites contained a benzoate daughter ion (m/z 121), indicating that the ring was unchanged by biotransformation. MEP, MBP and MHBP all fragmented with the loss of the free carboxy group to produce daughter ions of m/z 149, 177 and 193 (very weak) respectively. However, little fragmentation of the side chain was evident which, in the case of MHBP, prevented the location of the site of side chain hydroxylation. Fragmentation of this metabolite occurred primarily

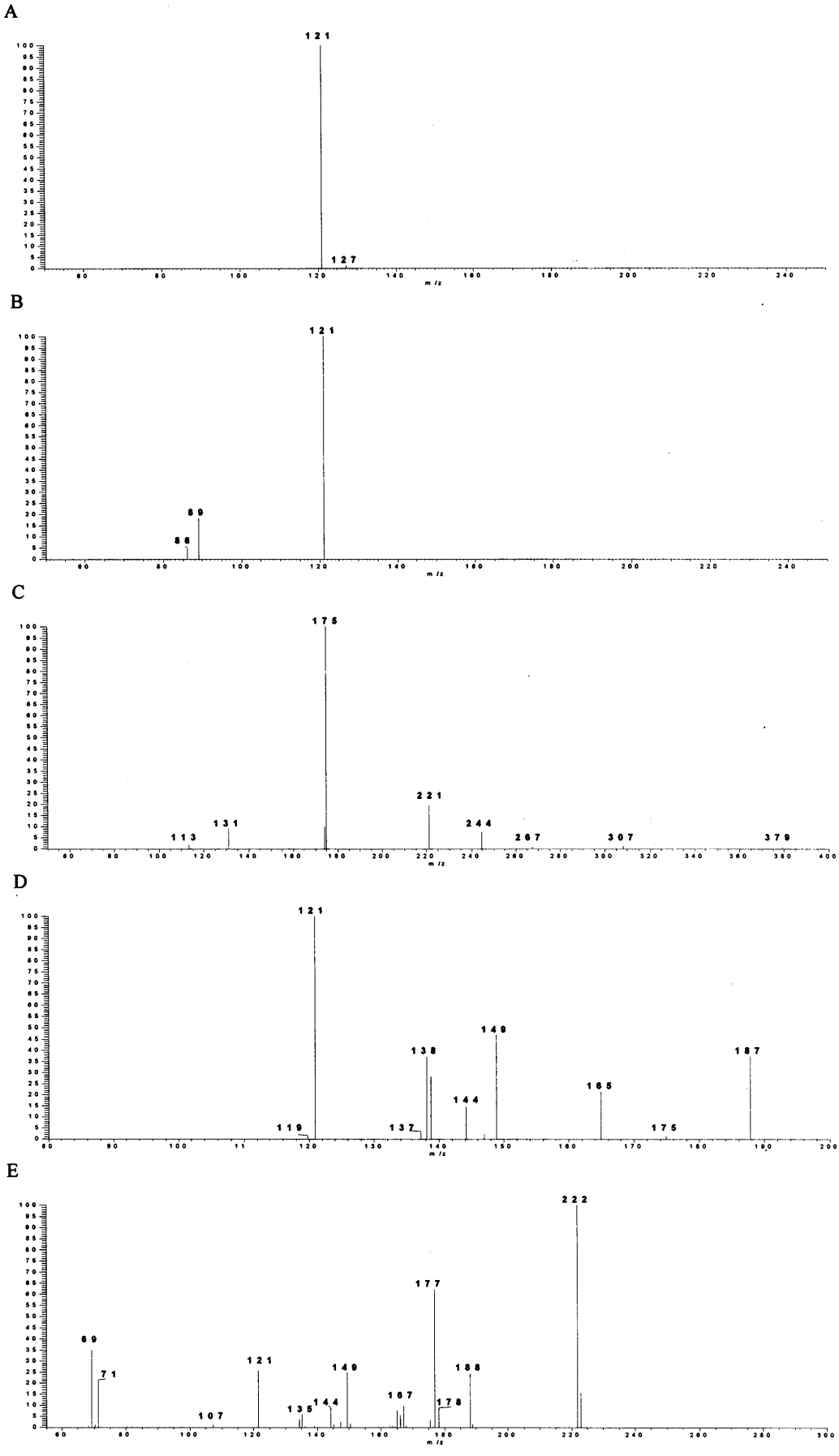

Figure 2. Representative daughter ion mass spectra (A–E) of DBP metabolites I–V respectively. In all cases spectra A–E were generated from M-H$^-$ of *m/z* 165, 237, 397, 193 and 221 respectively, with a CID of 26%.

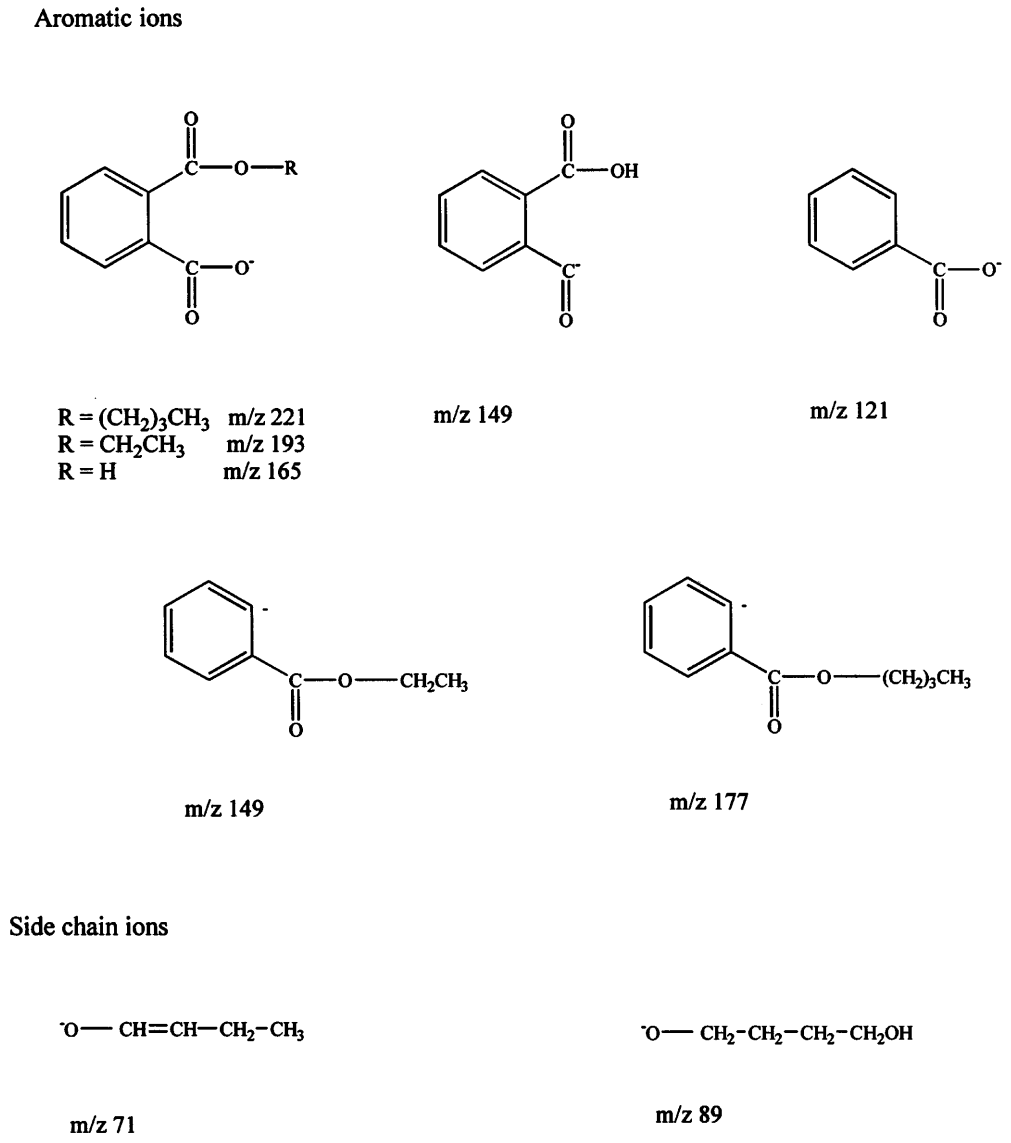

Figure 3. Structures of major ions present in the daughter ion mass spectra of metabolites I–V. Two structures are presented for daughter ion of *m/z* 149 found in spectra D and E.

through cleavage of the ester bond producing benzoate and n-hydroxybutoxy (*m/z* 89) daughter ions. Spectra of metabolite III contained daughter ions derived from glucuronic acid (*m/z* 175 and 113) and MBP (*m/z* 221). Grand-daughter ions of the aglycone (*m/z* 221) were constistent with daughters of reference MBP, providing clear evidence that this metabolite was MBP glucuronide. Similar CID of glucuronic acid conjugates occurs in triple sector quadrupole instruments during MS-MS experiments, yielding daughter ions derived from glucuronic acid and the aglycone.[25]

The structural information from mass spectral analysis was sufficient to determine the pathway of DBP biotransformation in cattle. In keeping with previous studies,[14–16] all biotransformations occurred in the side chain. The primary route of DBP metabolism, common to other species,[17] was hydrolysis of an ester bond to produce MBP, the major metabolite. Phthalic acid, formed by hydrolysis of a monoester, was a minor metabolite in bile and urine. In species such as the rat[14,16] a range of ω and ω-1 oxidation products of MBP, including monobutan-3-one, mono-3 and 4-hydroxybutyl and monobutanoic acid phthalates, have been found. In contrast, the range of ω-oxidation metabolites in cattle was limited to MHBP. Candidate biotransformation reactions for production of MEP, a novel metabolite of DBP, include either transethylation of MBP and MHBP or ethylation of phthalic acid. The low abundance of phthalic acid in this and other studies suggests that transethylation by a non-specific liver carboxyl esterase is most probable. Although not commonly reported, transethylation and ethylation are involved in the biotransformation of xenobiotics including cocaine[26] and acitretin[27], respectively. Phthalate alkyl side chains with six or more carbon atoms[17] may also be degraded by β-oxidation with the loss of a two carbon fragment. In the present study, neither a substrate nor product (in both cases an aliphatic acid phthalate) of such metabolism were found, indicating that the four carbon side chain was resistant to β-

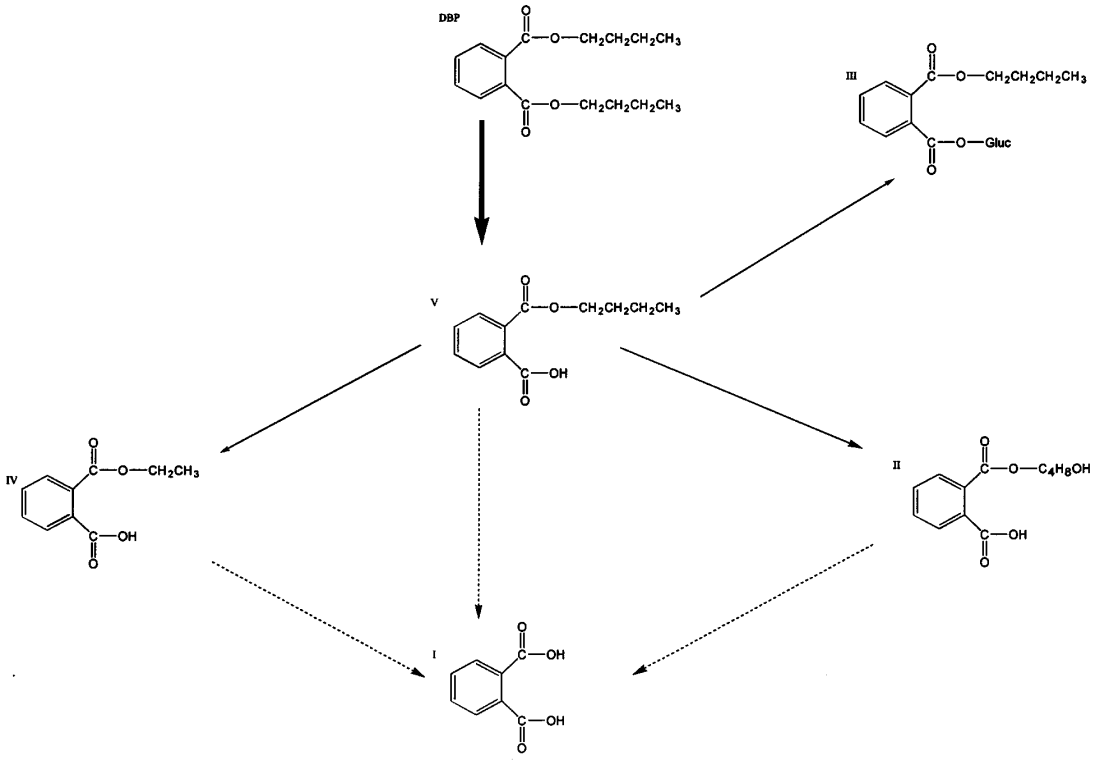

Figure 4. Proposed pathway for DBP biotransformation in the bovine. Bold and broken arrows indicate major and minor routes of metabolism respectively. Gluc indicates glucuronide, I phthalic acid, II MHBP, III MBP glucuronide, IV MEP and V MBP.

oxidation. Phase II metabolism in the bovine was limited to the formation of MBP glucuronide. Glucuronidation of MBP and related ω-oxidation products is common to most other species with the exception of the rat.[17] A pathway for biotransformation of DBP in the cattle is presented in Fig. 4.

Isolated bovine hepatocytes produced a similar profile of metabolites to those found in bile and enabled corroboration that the major metabolite in bile was MBP. Hepatocyte culture medium provides a relatively clean matrix for the identification of M-H$^-$ ions of metabolites in MS^1 spectra since the confounding influence of endogenous molecules, present at high concentrations in more complex matrices, such as bile, were absent. Such *in vitro* biotransformation studies enable the development of analytical methods for metabolite identification which can subsequently be applied to more complex matrices containing lower concentrations of analytes and corroboration of metabolite identity where analysis is problematic.

DBP was extensively biotransformed in cattle to a range of water soluble metabolites prior to excretion. Further studies with lactating dairy cattle are required to confirm the presence of these metabolites in edible tissues, or their transfer to products such as milk. Such confirmation will enable more complex analyses of edible tissues and dairy products for the presence of DBP and metabolite residues. This will provide for the identification of the route of food contamination with PAEs and, as a consequence, better targetting of appropriate remedial action to maintain high standards of consumer safety.

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