Synthesis and PMR and Mass Spectra of Potential Metabolites and Other Derivatives of Bis(2-ethylhexyl) Phthalate

JAMES E. CARTER, ROBERT V. PETERSEN, and DAVID B. ROLL ˣ

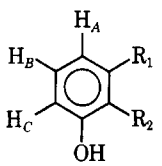
Abstract □ The 3- and 4-hydroxylated derivatives of the plasticizer bis(2-ethylhexyl) phthalate and the monoesters derived from these hydroxylated derivatives were synthesized. The structures of the two 4-hydroxy monoesters were confirmed by lithium borohydride reduction to their corresponding hydroxymethylbenzoic acids, followed by cyclization of the reduced compounds to known phthalides. All compounds were characterized by PMR and mass spectra. The PMR data reiterate the difficulty in predicting chemical shifts in multisubstituted benzene rings.

Keyphrases □ Phthalate esters, mono- and bis(2-ethylhexyl)—3- and 4-hydroxy derivatives synthesized and characterized by PMR and mass spectra □ Hydroxyphthalate esters, mono- and bis(2-ethylhexyl)—synthesized and characterized by PMR and mass spectra □ PMR—characterization of mono- and bis(2-ethylhexyl) esters of 3- and 4-hydroxyphthalic acid □ Mass spectrometry—characterization of mono- and bis(2-ethylhexyl) esters of 3- and 4-hydroxyphthalic acid

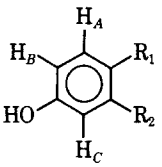
It has been shown that bis(2-ethylhexyl) phthalate (I) is converted into the corresponding monoester, mono(2-ethylhexyl) phthalate (II), both *in vivo* (1) and *in vitro* (2) followed by ω and ω-1 oxidation of II (3). Since II is 50–100 times more toxic than I (4), as determined by acute LD_{50} studies in mice and rats, other toxic metabolites, such as those formed by aromatic hydroxylation of I, might be formed in the body. In conjunction with studies aimed at the identification of such metabolites, the 3-hydroxy (III*a* and III*b*) and 4-hydroxy (IV*a*–IV*c*) derivatives of I and II and related compounds necessary for their unequivocal structural elucidation were synthesized and characterized by PMR and mass spectra.

EXPERIMENTAL

Instrumentation—PMR spectra[1] were obtained using dimethyl sulfoxide-d_6[2] as the solvent and sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard and are reported in δ, parts per million (ppm), relative to the internal standard. Mass spectra[3] were obtained by direct probe operating at 70 ev with an accelerating voltage of 3.5 kv and an ion source temperature of 250°.



III*a*: $R_1 = R_2 = COOCH_2CH(C_2H_5)(CH_2)_3CH_3$
III*b*: $R_1 = COOCH_2CH(C_2H_5)(CH_2)_3CH_3$, $R_2 = COOH$



IV*a*: $R_1 = R_2 = COOCH_2CH(C_2H_5)(CH_2)_3CH_3$
IV*b*: $R_1 = COOH$, $R_2 = COOCH_2CH(C_2H_5)(CH_2)_3CH_3$
IV*c*: $R_1 = COOCH_2CH(C_2H_5)(CH_2)_3CH_3$, $R_2 = COOH$

Melting points were determined using a micro hot stage or, where specified, a capillary melting-point apparatus[4] and are uncorrected. The TLC solvent system employed was chloroform–methanol–acetic acid (250:10:1).

For the PMR spectra, the aromatic protons in both the 3-hydroxy (III*a* and III*b*) and 4-hydroxy (IV*a*–IV*c*) derivatives were designated H_A, H_B, and H_C. The PMR data for the relevant protons of the 4-hydroxy derivatives are shown in Table I.

Mono(2-ethylhexyl) Phthalate (II)—This compound was synthesized from phthalic anhydride[5] and 2-ethylhexanol[5] according to the procedure of Kenyon and Platt (5); PMR: δ 7.65 (m, 4), 4.17 (d, 2), and 0.77–1.58 (m, 15) ppm; mass spectrum[6]: *m/e* 278 (M^+, 0.5%), 279 (M + 1, 7.5), 167 (30), 149 ($C_8H_5O_3$, 100), 113 (6), 112 (13), 104 (14), 57 (29), and 43 (55).

Bis(2-ethylhexyl) 4-Hydroxyphthalate (IV*a*)—To 3 g (16.4 mmoles) of 4-hydroxyphthalic acid[5] (V*a*) were added 33.4 g (256 mmoles) of 2-ethylhexanol and 3 drops of concentrated sulfuric acid dissolved in 75 ml of toluene (Scheme I, R = 2-ethylhexyl). The mixture was refluxed for 16 hr, the sulfuric acid was removed by shaking with 5% $NaHCO_3$, and the organic phase was washed with water. Toluene was removed with a flash evaporator, and 2-ethylhexanol was removed by vacuum distillation, leaving 5 g (75%) of crude liquid. The product was purified by silica gel[7] dry column chromatography (3.0 × 15 cm) (6) employing chloroform as the eluent; IV*a* eluted mainly in the first 100 ml, which then was concentrated and placed over a second silica gel dry column (1.2 × 20 cm). Elution with chloroform and solvent removal resulted in a clear viscous liquid of analytical quality; mass spectra: *m/e* 406 (M^+, 0.8%), 295 (21), 183 (43) m* 113.5, 165 (100) m* 148.8, 113 (9.5), 112 (20), 71 (25), 70 (30), 59 (29), and 43 (25).

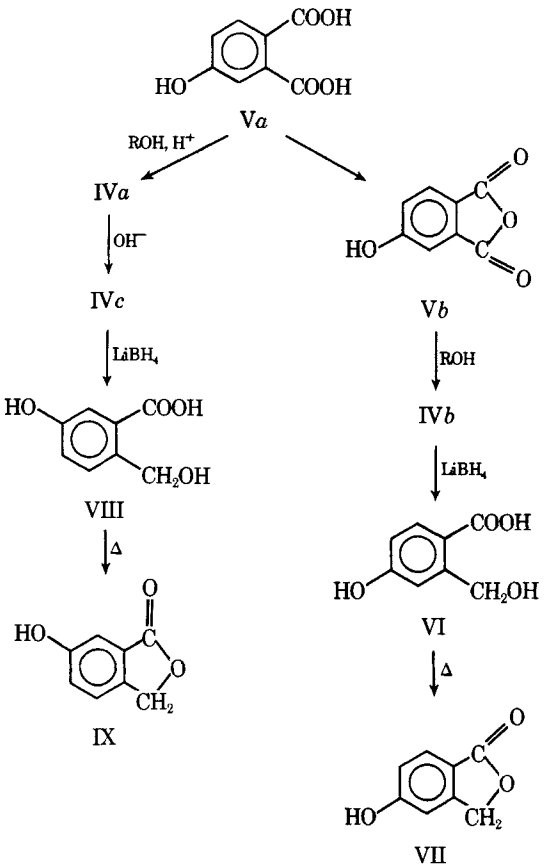
Anal.[8]—Calc. for $C_{24}H_{38}O_5$: C, 70.90; H, 9.42. Found: C, 70.71; H, 9.18.

2-(2-Ethylhexyl) 4-Hydroxyphthalate (IV*b*)—Two grams (10.9 mmoles) of V*a* was sublimed under vacuum to 4-hydroxyphthalic anhydride (V*b*) (1.69 g, 94%) and refluxed for 6 hr with 1.2 equivalents (1.6 g, 13 mmoles) of 2-ethylhexanol in 20 ml of toluene (Scheme I, R = 2-ethylhexyl). The reaction mixture contained only one product, as shown by TLC. The solution was filtered while hot. On cooling, the product crystallized in 70% (2.1 g) yield, mp 142–144°, when recrystallized from acetone–hexane; mass spectra: *m/e* 294 (M^+, 0.3%), 295 (M + 1, 0.9), 183 (10), 165 (50), 164 (82.5), 120 (100), 112 (19), 98 (28), 92 (25), 84 (30), 70 (40), 69 (35), and 57 (90).

Anal.—Calc. for $C_{16}H_{22}O_5$: C, 65.29, H, 7.53. Found: C, 65.51; H, 7.62.

1-(2-Ethylhexyl) 4-Hydroxyphthalate (IV*c*)—Two grams (10.9 mmoles) of V*a*, 8.2 g (62.9 mmoles) of 2-ethylhexanol, and 2 drops of concentrated sulfuric acid were refluxed in 20 ml of toluene for 16 hr and yielded IV*a*, which was hydrolyzed by refluxing for 4 hr after the addition of 2 g of sodium hydroxide (Scheme I). In addition to the desired product (IV*c*), small amounts of IV*b* and V*a* were shown to be present by silica gel[9] TLC. The reaction mixture was acidified with 4 *N* sulfuric acid and extracted with ether, and the product was purified by passage through a silica gel dry column (1.5 × 16 cm). The column was eluted with 100 ml of chloroform followed by increasing concentrations of methanol in chloroform, resulting in elution of the product (1.1 g, 34%) with 25% methanol, mp 124–126° from acetone–hexane; mass spectra: *m/e* 294

[1] JNM-C-60 spectrometer.
[2] Stohler Isotope Chemicals.
[3] LKB 9000S.
[4] A. H. Thomas.
[5] Eastman Chemical Co.
[6] The m* designates metastable ions.
[7] Brinkmann, 0.05–0.20 mm.
[8] Heterocyclic Chemical Corp.
[9] Mallinckrodt Chemical Works.



Scheme I

(M+, 0.3%), 295 (M + 1, 1.0), 183 (19.5), 165 (95), 164 (20.0), 120 (40), 92 (28), 83 (26), 70 (45), 57 (100), 43 (35), and 41 (33).

2-Hydroxymethyl-4-hydroxybenzoic Acid (VI)—To confirm the structure of IV*b*, VI was prepared by refluxing IV*b* (0.81 g, 2.77 mmoles) for 24 hr with a 10-fold excess of lithium borohydride[10] (0.60 g, 28 mmoles) in 30 ml of dioxane (Scheme I). The reaction mixture was acidified with 2 *N* hydrochloric acid and extracted with ether, and the ether was removed under vacuum. The product (0.27 g, 58%), recrystallized from methanol–water, slowly sublimed between 140 and 170°, probably forming the corresponding lactone but never giving a sharp melting point; mass spectra: *m/e* 168 (M+, 4%), 150 (70), 121 (100) m* 97.6, 93 (19) m* 71.5, and 65 (21) m* 45.4.

2-Hydroxymethyl-4-hydroxybenzolactone (VII)—To aid in the identification of IV*b*, VI was converted quantitatively to its corresponding lactone (VII) by heating at 100° for 72 hr (Scheme I), mp 219° with softening at 210° (capillary) [lit. (7) mp 223° with softening at 210°]; mass spectra: *m/e* 150 (M+, 74%), 121 (100) m* 97.6, 93 (21) m* 71.5, and 65 (25) m* 45.4.

2-Hydroxymethyl-5-hydroxybenzoic Acid (VIII)—This compound was prepared by refluxing 2.5 g of IV*c* (8.5 mmoles) with 1.85 g (85 mmoles) of lithium borohydride in dioxane for 36 hr (Scheme I). Excess borohydride was destroyed by the addition of 2 *N* hydrochloric acid, the solution was extracted with ether and dried with sodium sulfate, and the ether was removed, giving the desired product (0.52 g, 36%). This product was recrystallized from methanol–water, mp 129–130° (capillary); mass spectra: *m/e* 168 (M+, 1.1%), 150 (46), 121 (100) m* 97.6, 93 (28) m* 71.5, and 65 (26) m* 45.4.

2-Hydroxymethyl-5-hydroxybenzolactone (IX)—Compound VIII was quantitatively converted to IX by heating at 100° for 6 hr (Scheme I), mp 199–200° (capillary) [lit. (7) mp 201–202°]; mass spectra: *m/e* 150 (M+, 57%), 121 (100) m* 97.6, 93 (35) m* 71.5, and 65 (25) m* 45.4.

3-Hydroxyphthalic Anhydride (X*c*)—3-Aminophthalic acid[5] (X*a*) (2.0 g, 11.0 mmoles) was diazotized with 0.76 g (11.0 mmoles) of sodium nitrite in 20 ml of 15 *N* sulfuric acid (Scheme II, R = 2-ethylhexyl) following the procedure of Eliel *et al.* (8). The resultant 3-hydroxyphthalic acid (X*b*) was sublimed *in vacuo* to the anhydride (X*c*) (0.71 g, 40%), mp 195–196° [lit. (9) mp 199–200°], and was used without further purification in the following syntheses.

Table I—Chemical Shift Values (δ) for Derivatives of 4-Hydroxyphthalic Acid (V*a*)

Compound	H_A	H_B	H_C	$-CH_2-$
V*a*	7.65 (d, $J \simeq 9$)	6.90	6.90	—
↓ IV*a*	7.66 (d, $J \simeq 9$)	6.95	6.90	4.10
↓ IV*c*	7.35 (d, $J \simeq 8$)	6.72 (dd, $J \simeq 2,8$)	7.00 (d, $J \simeq 2$)	4.05
↓ VIII	7.47 (d, $J \simeq 8$)	6.94 (dd, $J \simeq 2,8$)	7.28 (d, $J \simeq 2$)	4.73
↓ IX	7.53 (d, $J \simeq 9$)	7.23	7.15	5.30
IV*b*	7.72 (d, $J \simeq 8$)	6.92	6.83	4.13
↓ VI	7.77 (d, $J \simeq 8$)	6.67 (dd, $J \simeq 2,8$)	7.12 (d, $J \simeq 2$)	4.82
↓ VII	7.67 (d, $J \simeq 9$)	6.97	6.97	5.27

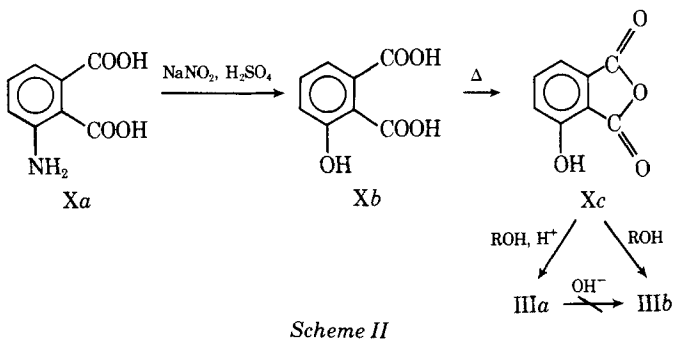
Bis(2-ethylhexyl) 3-Hydroxyphthalate (III*a*)—Compound X*c* (1.0 g, 6.1 mmoles) was added to 20 ml of 2-ethylhexanol (16.7 g, 128.5 mmoles) in 30 ml of toluene plus 3 drops of concentrated sulfuric acid and refluxed for 16 hr (Scheme II, R = 2-ethylhexyl). The reaction mixture was treated similarly to that in the synthesis of IV*a*. The crude product (1.5 g, 60%) was purified for analysis by silica gel dry column chromatography (1.4 × 15 cm) by elution with hexane followed by increasing concentrations of chloroform in hexane. Fractions containing III*a* were concentrated and placed over a neutral alumina[11] dry column (1.2 × 20 cm), which was eluted with benzene followed by 25% chloroform in benzene. Removal of the solvent yielded a clear, viscous liquid; PMR: δ 7.68 (dd, H_A), 6.98 (m, H_B and H_C), 4.15 (d, 4), and 0.77–1.58 (m, 30) ppm; mass spectra: *m/e* 406 (M+, 7.5%), 295 (9), 294 (10.5), 183 (31.5), 182 (28.5), 165 (100), 164 (29), 149 (24), 113 (30), 112 (56), and 71 (47.5).

Anal.—Calc. for $C_{24}H_{38}O_5$: C, 70.90; H, 9.42. Found: C, 71.02; H, 9.86.

1-(2-Ethylhexyl) 3-Hydroxyphthalate (III*b*)—This compound was synthesized from X*c* (0.71 g, 4.3 mmoles) and 2-ethylhexanol (1.1 g, 8.6 mmoles) by refluxing in 20 ml of pyridine for 8 hr (Scheme II, R = 2-ethylhexyl). The reaction mixture was acidified to pH 3 with 4 *N* sulfuric acid, extracted with ether, and then shaken with 50 ml of 5% Na_2CO_3. The basic solution was carefully acidified, resulting in the precipitation of III*b* (0.85 g, 72%), mp 191–192° when recrystallized from acetone–hexane. TLC indicated that only one isomer was formed (R_f 0.51). Attempts to synthesize the other isomer of III*b* by hydrolysis of III*a* (analogous to hydrolysis of IV*a* to IV*c*) were unsuccessful. Compound III*b* is probably the 1-(2-ethylhexyl) ester; PMR: δ 7.20 (dd, H_A), 6.88 (m, H_B and H_C), 4.08 (d, 2), and 0.77–1.58 (m, 15) ppm; mass spectra: *m/e* 294 (M+, 1.3%), 221 (32), 183 (2.5), 182 (3.5), 165 (20), 164 (100), 120 (95), 112 (36), 92 (90), 83 (86), and 70 (60).

RESULTS AND DISCUSSION

Chemistry—Scheme I shows the reactions involved in the synthesis of the various derivatives of 4-hydroxyphthalic acid (V*a*). The reaction



Scheme II

[10] ICN, Inc.

[11] Woelm, Waters Associates.

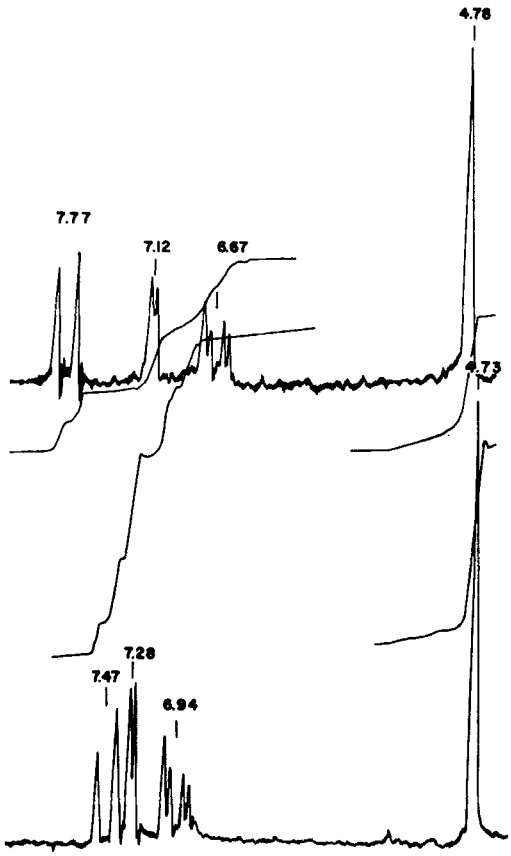


Figure 1—*PMR spectra of the hydroxymethylbenzoic acids VI (upper) and VIII (lower).*

of 4-hydroxyphthalic anhydride (V*b*) and 2-ethylhexanol yielded only one isomer, subsequently shown to be IV*b*. Alkaline hydrolysis of IV*a*, however, resulted mainly in the formation of the geometric isomer, IV*c*; small amounts of phthalic acid and IV*b* also were obtained. The results in both cases are explicable when one considers that the 2-ethylhexanol or the hydroxide ion in the two reactions, respectively, would be most likely to attack the more electropositive carbonyl group, which is the one *meta* to the phenolic hydroxyl in both cases.

Scheme II shows the reaction pathway for the synthesis of the derivatives of 3-hydroxyphthalic acid (X*b*). Although the position of the ester group in the 3-hydroxy monoester (III*b*) was not unequivocally established, the electron-donating effect of the hydroxyl group to the *ortho*-position suggests that the 1-(2-ethylhexyl) ester is the compound formed under the reaction conditions employed. There was no evidence by TLC of formation of the isomer of III*b*, even when III*a* was subjected to hydrolysis in base, a procedure successful in the conversion of IV*a* to IV*c*. Regardless of the preparation method of III*b*, the products isolated had identical PMR and mass spectra. Compound III*b* was the only hydroxylated metabolite subsequently found on incubation of liver homogenates[12].

Lithium borohydride was chosen as the reducing agent for IV*b* and IV*c*, since this reagent selectively reduces esters in the presence of carboxylic acids (10). A large excess of lithium borohydride was required for quantitative reduction because of the ionization of the carboxylic acid and consequent insolubility of the complex. The hydroxymethylbenzoic acids formed (VI and VIII) were then cyclized to their known lactones. This conversion was quite facile, particularly in the case of IX, since it was formed when the reaction mixture for the synthesis of VIII from IV*c* was made too acidic when destroying the excess borohydride.

Attempted reduction of III*b* with lithium borohydride to distinguish unequivocally the position of the 2-ethylhexyl group was unsuccessful.

PMR Spectra—The relevant PMR data for the various derivatives of 4-hydroxyphthalic acid are shown in Table I. The assignment of the signals of H_A, H_B, and H_C were made by their coupling constants and chemical shifts. Whereas the signal for H_A in V*a* (δ 7.65) and IV*a* (δ 7.66) had nearly identical chemical shifts, the same signal in two monoesters, IV*b* (δ 7.72) and IV*c* (δ 7.35), showed a significant difference. From these data, it became obvious that the unequivocal structural assignment of IV*b* and IV*c* could not be made on the basis that H_A was adjacent to the acid in one isomer and the ester in the other. Although it is difficult to predict the chemical shift of a given proton on multiple-substituted benzene rings (11), it was felt that selective reduction of the ester carbonyl of IV*c* to the corresponding hydroxymethyl compound (VIII) would result in a significant upfield shift of H_A and that a similar reduction of the ester group of IV*b* would result in little change in the chemical shift of H_A.

Similarly, the signal of H_C in VIII would be expected to remain relatively unchanged compared to that of H_C in IV*c*, and the signal of H_C in VI might be expected to move upfield compared to that of IV*b*; in the latter case, H_C would be *ortho* to the hydroxymethyl group instead of the deshielding carbonyl moiety. From an examination of Table I, it is apparent that, upon reduction of IV*b* and IV*c*, there was an unexpected small downfield shift in the signal of H_A in both cases whereas there was a more significant downfield shift of 0.29 and 0.28 ppm in the signal of H_C of the reduced products, VI and VIII, respectively.

The PMR spectra of VI and VIII are shown in Fig. 1. The assignments of H_A in VI (upper spectrum, δ 7.77) and VIII (δ 7.47) were made on the basis of their being furthest downfield and their coupling constants of 8 Hz. The signal of H_C was assigned at δ 7.12 and 7.28 in VI and VIII, respectively, by the small *meta*-coupling of 2 Hz; the signal of H_B was the furthest upfield in both cases, appearing as a doublet of doublets with coupling constants of about 8 and 2 Hz, respectively.

Although the PMR data for the reduction of IV*b* and IV*c* were not entirely expected, as expected the signals of H_A and H_C were at lower fields when adjacent to a carbonyl group than when adjacent to a hydroxymethyl group; *i.e.*, the signal of H_A was downfield in VI compared to VIII and, similarly, the signal of H_C was downfield in VIII compared to VI (Table I). Unequivocal proof of structure of the monoesters IV*b* and IV*c* was obtained by conversion of VI and VIII to their corresponding known lactones, VII and IX, respectively, which were characterized by their melting points and PMR and mass spectra. The most noteworthy feature of the PMR spectra of these compounds was that the signal for the methylene protons was downfield approximately 0.5 ppm from the analogous signals in VI and VIII. This downfield shift was consistent with that observed for the methylene protons of alcohols and their corresponding esters (12).

The PMR spectra of the 3-hydroxyphthalates, III*a* and III*b*, were unremarkable but consistent with the structures assigned.

Mass Spectrometry—The mass spectral data for II and IV*a*–IV*c* were consistent with the assigned structures. One of the most characteristic fragments for phthalate esters occurred at *m/e* 149 (13), and this fragment was the base peak for II. Since IV*a*–IV*c*, III*a*, and III*b* are hydroxyphthalates (with 16 additional amu), the main fragment for these compounds appeared at *m/e* 165. For diesters such as IV*a*, the most characteristic fragmentation was the loss of the alkyl fragment with transfer of one or two hydrogen atoms to produce the carboxylic acid ion or the protonated carboxylic acid ion, respectively (14). In the case of IV*a*, the protonated carboxylic acid ion (*m/e* 295) was formed, which then lost a second 2-ethylhexyl fragment in a typical McClafferty reaction (15) to form the protonated diacid (*m/e* 183). This transition, although uncomplicated, was also supported by the metastable ion[13] at *m/e* 113.5. Loss of water gave a peak at *m/e* 165 (again consistent with a metastable ion at *m/e* 148.8) and was followed by loss of CO_2 (*m/e* 121). Fragments between 41 and 113 amu mostly arose from fragmentation of the 2-ethylhexyl side chain.

The mass spectra of IV*b* and IV*c* were similar and explicable, except that an artifact appeared for both compounds at *m/e* 295, 1 amu higher than the expected molecular ion *m/e* 294. Such an artifact also appeared in the mass spectrum of II. This unexpected peak was shown to be the result of a bimolecular collision, with a fragment ion donating a proton to the molecular ion. The abundance of the M + 1 species was dependent upon the sample pressure in the ion source. The proper identification of the molecular ion and of M + 1 as an artifact was accomplished by scanning the total ion current peak and noting the disappearance of M + 1 as the sample pressure decreased.

The mass spectra of the 3-hydroxyphthalates were consistent with the structures assigned. In the case of the monoester III*b*, the formation of both the carboxylic acid ion (*m/e* 182) and the protonated carboxylic acid ion (*m/e* 183) was apparent, but the formation of the base peak at *m/e*

[12] Unpublished data.

[13] Metastable peaks (m*) are formed when an ion, m, decomposes during flight through the instrument and is analyzed with a mass m_2. The masses are related by the expression $m^* = (m_2^2/m_1)$ (16).

164 (182 − H_2O) indicated that the former fragmentation path predominated.

The mass spectral data for the reduced products VI and VIII and their corresponding lactones, although unremarkable, were compatible with the assigned structures. As might be expected for the hydroxymethylbenzoic acids, a major fragment at *m/e* 150 indicated the loss of water with cyclization to the corresponding lactones.

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[x] To whom inquiries should be directed.

Bioavailability of Ampicillin and Amoxicillin in Fasted and Nonfasted Subjects

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Abstract □ The influence of various test meals and fluid volume on the relative bioavailability of ampicillin and amoxicillin was studied in healthy human subjects. Serum amoxicillin levels were somewhat, but not always, significantly higher than those of ampicillin from equivalent oral doses. Food ingested immediately before dosing reduced serum levels and urinary excretion of both antibiotics to a similar extent. Reduction of dosed water volume caused a marked decrease in serum amoxicillin levels in fasted subjects.

Keyphrases □ Ampicillin—bioavailability, oral administration, effect of fasting and fluid volume, humans □ Amoxicillin—bioavailability, oral administration, effect of fasting and fluid volume, humans □ Bioavailability—ampicillin and amoxicillin, oral administration, effect of fasting and fluid volume, humans □ Antibacterial agents—ampicillin and amoxicillin, bioavailability, oral administration, effect of fasting and fluid volume, humans

Ampicillin and amoxicillin have similar antibacterial activity against various organisms (1). Amoxicillin has twice the activity of ampicillin against enterococci and *Salmonella* species but is somewhat less active than ampicillin against *Haemophilus* and *Shigella* species (2, 3).

Various reports indicated that, despite the similar antibacterial spectrum of the two compounds, amoxicillin has superior bioavailability properties from oral dosage forms and may, therefore, be the compound of choice when this route of administration is used (4–6). Amoxicillin absorption may be less influenced by food than ampicillin absorption, so less variation in circulating antibiotic levels might be expected during repeated oral doses of amoxicillin (7, 8).

In this study, the bioavailability of ampicillin and amoxicillin was compared in fasted and nonfasted subjects under carefully controlled conditions.

EXPERIMENTAL

The subjects were three male and three female healthy volunteers. Male subjects were 24–30 years old (mean 27) and weighed 64–81 kg (mean 74). Female subjects were 21–27 years old (mean 23) and weighed 50–68 kg (mean 60). All subjects were shown by medical examination to be in good physical condition with normal blood and urine laboratory values. The subjects had no histories of allergic reaction to penicillins.

Protocol—Verbal assurance was obtained from all subjects that they had taken no known enzyme-inducing agents for 1 month and no other drugs for 1 week preceding the study. Subjects were instructed to take no drugs other than the required doses of antibiotic during the study.

The subjects were fasted overnight before each treatment and were permitted to eat no food, apart from test meals, until 4 hr after dosing. On the morning of a treatment, each subject drank 250 ml of water on arising, at least 1 hr before dosing. Medication was administered at 8 am; blood samples (4–5 ml) were collected from a forearm vein into vacuum tubes[1] containing no anticoagulant immediately before dosing and at 20 and 40 min and 1, 1.5, 2, 3, 4, 6, and 8 hr after dosing. Serum was separated and deep frozen at −18° until assayed. Urine was collected through 8 hr

[1] Vacutainers.