

Sci. Ed., 48, 431 (1959).

(15) R. B. Bell and F. J. Lindlars, *J. Chem. Soc.*, 1954, 4601.

(16) E. R. Garrett, *J. Am. Chem. Soc.*, 79, 5206 (1957).

(17) H. V. Maulding and M. A. Zoglio, *J. Pharm. Sci.*, 59, 700 (1970).

(18) R. B. Martin, R. I. Heddrick, and A. Parcell, *J. Org. Chem.*, 29, 3197 (1964).

(19) R. E. Barnett and W. P. Jencks, *J. Am. Chem. Soc.*, 91, 2358 (1969).

(20) R. E. Notari, M. L. Chin, and A. Cardoni, *J. Pharm. Sci.*, 59, 28 (1970).

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Quantification of Lidocaine and Several Metabolites Utilizing Chemical-Ionization Mass Spectrometry and Stable Isotope Labeling

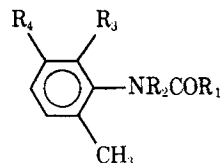
S. D. NELSON, W. A. GARLAND, G. D. BRECK, and W. F. TRAGER *

Abstract □ Quantification of the suspected metabolites of lidocaine in humans was carried out using the direct insertion probe and chemical-ionization mass spectrometry. Deuterated analogs of the metabolites of lidocaine were added to serial human plasma and urine samples and were used as internal standards following oral administration of 250 mg of lidocaine hydrochloride monohydrate to two male subjects and 202 mg of lidocaine free base to one male subject. The average results after analysis of the 0–24-hr urine samples, before β -glucuronidase-sulfatase treatment, indicated the presence of seven of the possible metabolites in the following amounts (percent of administered dose based on the free base): lidocaine, 1.95; ω -ethylamino-2,6-dimethylacetanilide, 4.90; ω -amino-2,6-dimethylacetanilide, 0.88; *m*- and/or *p*-hydroxylidocaine, 0.73; *m*- and/or *p*-hydroxy- ω -ethylamino-2,6-dimethylacetanilide, 0.56; 2,6-dimethylaniline, 0.97; and 4-hydroxy-2,6-dimethylaniline, 63.5. Both *N*-ethyl- and *N,N*-diethylglycine were detected in human and Rhesus monkey urine, although quantification was not achieved.

Keyphrases □ Lidocaine and various metabolites—chemical-ionization mass spectrometric and stable isotope labeling analyses, human plasma and urine □ Chemical-ionization mass spectrometry—analyses, lidocaine and various metabolites, human plasma and urine □ Stable isotope labeling—analyses, lidocaine and various metabolites, human plasma and urine □ Anesthetics, local—lidocaine and various metabolites, chemical-ionization mass spectrometric and stable isotope labeling analyses, human plasma and urine

Lidocaine (Ia) is a widely used local anesthetic and antiarrhythmic agent. At present, it is generally considered the most useful drug in suppressing ventricular arrhythmias, which occur in approximately 80% of all myocardial infarction cases (1–3). Based on the structure of the drug, three sites are susceptible to metabolic attack: the tertiary amino group, the amide linkage, and the aromatic ring. The tertiary amino group is susceptible to *N*-oxidation and oxidative *N*-dealkylation (4). The amide linkage is susceptible to hydrolysis by microsomal amidases (5) and to oxidation by liver microsomes to an *N*-hydroxyamide (4). The aromatic ring and the aryl methyl groups are also susceptible to hydroxylation (4). Many of these reactions were detected using nonspecific colorimetric assays and paper chromatography (6–9) and, more recently, more specific GC methods (10–12).

Using electron-impact mass spectrometry, Breck and Trager (13) confirmed the presence of lidocaine, ω -ethylamino-2,6-dimethylacetanilide (Ib), and 2,6-dimethylaniline (IIa) in human urine following an oral dose or an intravenous infusion of lidocaine. They also detected small amounts of a new cyclic metabolite of lidocaine, *N*¹-ethyl-2-methyl-*N*³-(2,6-dimethylphenyl)-4-imidazolidinone (III). GC-mass spectrometry was used to detect and quantify lidocaine and its two *N*-deethylated metabolites, Ib and ω -amino-2,6-dimethylacetanilide (Ic), in the plasma and urine of patients receiving intravenous infusions of the drug for the treatment of cardiac arrhythmias (14, 15). Under these conditions, lidocaine levels ranged from 1.2 to 15 μ g/ml, Ib levels ranged from 0.2 to 2.4 μ g/ml, and Ic levels ranged from <1.0 to 2.7 μ g/ml, with



Ia: $R_1 = \text{CH}_2\text{N}(\text{CH}_2\text{CH}_3)_2$, $R_2 = \text{H}$, $R_3 = \text{CH}_3$, $R_4 = \text{H}$

Ia-d₄: $R_1 = \text{CH}_2\text{N}(\text{CD}_2\text{CH}_3)_2$

Ib: $R_1 = \text{CH}_2\text{NHCH}_2\text{CH}_3$, $R_2 = \text{H}$, $R_3 = \text{CH}_3$, $R_4 = \text{H}$

Ib-d₃: $R_1 = \text{CH}_2\text{NHCH}_2\text{CD}_3$

Ic: $R_1 = \text{CH}_2\text{NH}_2$, $R_2 = \text{H}$, $R_3 = \text{CH}_3$, $R_4 = \text{H}$

Ic-d₂: $R_1 = \text{CD}_2\text{NH}_2$

Id: $R_1 = \text{CH}_2\text{N}(\text{CH}_2\text{CH}_3)_2$, $R_2 = \text{H}$, $R_3 = \text{CH}_3$, $R_4 = \text{OH}$

Id-d₄: $R_1 = \text{CH}_2\text{N}(\text{CD}_2\text{CH}_3)_2$

Ie: $R_1 = \text{CH}_2\text{NHCH}_2\text{CH}_3$, $R_2 = \text{H}$, $R_3 = \text{CH}_3$, $R_4 = \text{OH}$

Ie-d₃: $R_1 = \text{CH}_2\text{NHCH}_2\text{CD}_3$

If: $R_1 = \text{CH}_2\text{N}(\text{CH}_2\text{CH}_3)_2$, $R_2 = \text{H}$, $R_3 = \text{CH}_2\text{OH}$, $R_4 = \text{H}$

Ig: $R_1 = \text{CH}_2\text{NHCH}_2\text{CH}_3$, $R_2 = \text{H}$, $R_3 = \text{CH}_2\text{OH}$, $R_4 = \text{H}$

Ig-d₂: $R_2 = \text{CD}_2\text{OH}$

Ih: $R_1 = \text{CH}_2\text{N}(\text{CH}_2\text{CH}_3)_2$, $R_2 = \text{OH}$, $R_3 = \text{CH}_3$, $R_4 = \text{H}$

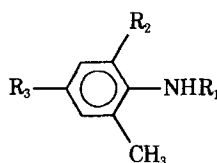
Ih-d₄: $R_1 = \text{CH}_2\text{N}(\text{CD}_2\text{CH}_3)_2$

Ii: $R_1 = \text{CH}_2\text{NHCH}_2\text{CH}_3$, $R_2 = \text{OH}$, $R_3 = \text{CH}_3$, $R_4 = \text{H}$

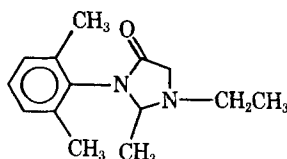
Ii-d₃: $R_1 = \text{CH}_2\text{NHCH}_2\text{CD}_3$

Ij: $R_1 = \text{CH}_2\text{N}(\text{CH}_2\text{CH}_3)_2$, $R_2 = \text{H}$, $R_3 = \text{CH}_3$, $R_4 = \text{H}$

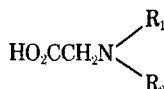
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IIa: $R_1 = H$, $R_2 = CH_3$, $R_3 = H$ IIa- d_2 : $R_2 = CHD_2$
 IIb: $R_1 = H$, $R_2 = CH_2OH$, $R_3 = H$ IIb- d_2 : $R_2 = CD_2OH$
 IIc: $R_1 = OH$, $R_2 = CH_3$, $R_3 = H$
 IId: $R_1 = H$, $R_2 = CH_3$, $R_3 = OH$ IId- d_2 : $R_2 = CHD_2$



III



IVa: $R_1 = CH_2CH_3$, $R_2 = CH_2CH_3$
 IVa- d_4 : $R_1 = CD_2CH_3$, $R_2 = CD_2CH_3$
 IVb: $R_1 = CH_2CH_3$, $R_2 = H$
 IVb- d_3 : $R_1 = CH_2CD_3$, $R_2 = H$

Ic levels $<1.0 \mu\text{g/ml}$ not measurable. A rapid, specific, analytical technique for monitoring these metabolites might be useful, since one or more of these biotransformation products may be responsible for some of the central nervous system toxicity associated with lidocaine therapy.

A more direct quantification of quinidine, lidocaine, and their metabolites in human plasma, utilizing a combination of stable isotope labeling and chemical-ionization mass spectrometry, was described previously (16). This technique is now applied to the quantification of lidocaine and several of its metabolites in human plasma and urine.

The chemical-ionization mass spectrometry-stable isotope technique, combined with some more classical techniques, confirmed (at least under the *in vivo* conditions of dosage and analysis used in this study) the presence of many suspected lidocaine metabolites and showed the absence of others. To carry out this analysis, the 17 suspected metabolites (Ia-Ij, IIa-IIj, III, IVa, and IVb) and the deuterated analogs were synthesized.

EXPERIMENTAL¹

Equipment—Melting points² are uncorrected. When obtaining chemical-ionization mass spectra, the electron gun voltage was set at 510 v and the ion repeller was set between 0.0 and 1.0 v. The source and analyzer ion gauges read 1.8×10^{-4} and 7.6×10^{-6} torr, respectively. The source chamber pressure was 0.5 torr as read from a calibrated McLeod gauge some distance from the ion chamber. Both sample and reagent gas were introduced *via* a specially designed direct insertion probe (17, 18).

All mass spectra were run at an accelerating voltage of 8 kv and a mass resolution of $m/\Delta = 3500$ (5% valley definition). All samples were dissolved in either ethanol³ or methanolic hydrogen chloride, the solution

was applied to the ceramic probe tip, and the solvent was evaporated with a gentle stream of nitrogen. Elemental analyses⁴ were obtained where appropriate.

Lidocaine (Ia)—Compound Ia⁵ was recrystallized from purified petroleum ether (bp 30–60°) to obtain colorless needles, mp 67–68° (19). The chemical-ionization mass spectrum showed that the MH^+ ion at m/e 235 and its ^{13}C -satellite represented 84.8% of the total ion current; m/e 164 represented 15.2% of the total ion current from m/e 100 to 300.

Lidocaine- d_4 (Ia- d_4)—A mixture of freshly distilled acetonitrile (4.10 g, 0.1 mole) and 200 mg of Adam's catalyst in freshly distilled acetic anhydride (30 ml) was hydrogenated (30 psi, deuterium gas) for 4 hr. An additional 100 mg of catalyst was added, and the hydrogenation was continued an additional 2 hr. After removal of reduced platinum by suction filtration, the filtrate was distilled through a short-path fractionating column to yield *N*-ethylacetamide- d_2 , bp 204–207° (7.1 g, 80%).

To an ice bath-cooled solution of aluminum trideuteride (~46 mmoles generated from lithium aluminum deuteride and sulfuric acid) in tetrahydrofuran was added dropwise a tetrahydrofuran solution of *N*-ethylacetamide- d_2 (1.90 g, 23 mmoles) over 60 min. The solution was slowly allowed to come to room temperature and was stirred an additional 18 hr under nitrogen. Excess aluminum trideuteride was destroyed with 4 ml of tetrahydrofuran–water (1:1), followed by hydrolysis of aluminate complexes with 8 ml of 15% NaOH. The liberated diethylamine- d_4 was codistilled with tetrahydrofuran at 59–60°.

To the tetrahydrofuran solution, 60 ml, containing diethylamine- d_4 was added ω -chloro-2,6-dimethylacetanilide (19) (1.97 g, 10 mmoles), and the solution was stirred at reflux for 3 hr. To the solution was added 450 mg of sodium hydroxide in 2 ml of water, and the reaction was refluxed an additional 2 hr. Rotary evaporation of solvent yielded a pale-yellow liquid, which was taken up in ether, washed with water, and extracted into 10% HCl. The acid extract was made basic with 5 *N* NaOH and back-extracted into ether. The ether back-extract was washed with water, filtered through anhydrous sodium sulfate, and dried over calcium sulfate⁶. Removal of solvent and three recrystallizations from petroleum ether yielded 1.4 g (59%), mp 65–67°; NMR: δ^T 1.13 [broad s, $N(CD_2CH_3)_2$], 2.23 (s, aryl methyls), 3.20 (s, $O=CCH_2N$), 7.07 (s, aromatics), and 8.90 (broad s, $HNC=O$) ppm; high-resolution mass measurement: calc. for $C_{14}H_{18}^2H_4N_2O$, 238.1983; found, 238.1979.

A chemical-ionization mass spectral analysis showed the following isotopic abundances: lidocaine- d_4 , 71.5% (m/e 239); lidocaine- d_3 , 19.0% (m/e 238); lidocaine- d_2 , 4.4% (m/e 237); lidocaine- d_1 , 5.1% (m/e 236); and lidocaine, 0% (m/e 235).

ω -(β - 2H -Ethylamino)-2,6-dimethylacetanilide (Ib- d_3)—Ethylamine- d_3 hydrochloride was synthesized, according to the procedure of Ross *et al.* (20), by lithium aluminum hydride reduction of acetonitrile- d_3 .

To a magnetically stirred mixture of ω -chloro-2,6-dimethylacetanilide (295 mg, 2 mmoles) and ethylamine- d_3 hydrochloride (254 mg, 3 mmoles) in dioxane (20 ml) and water (5 ml) was added anhydrous potassium carbonate (420 mg, 3 mmoles) in 20-mg portions over 2 hr while the reaction was heated at 60° in an oil bath. After the addition was complete, the reaction was stirred an additional 3 hr. Rotary evaporation of the solvent yielded a white crystalline mass, which was worked up as described for Ib. Removal of solvent gave a pale-yellow liquid, which was taken up in 20 ml of purified hexane and recrystallized to yield 350 mg (80%) of small needles, mp 49–51°. The hydrochloride was made by dissolving the free base in absolute ethanol and bubbling through anhydrous hydrogen chloride. Light needles formed and were collected by suction filtration, mp 285–288° dec.

A chemical-ionization mass spectral analysis showed the following isotopic abundances: Ib- d_3 , 66.0% (m/e 210); Ib- d_2 , 22.4% (m/e 209); Ib- d_1 , 8.0% (m/e 208); and Ib, 3.6% (m/e 207).

ω -Amino-2,6-dimethylacetanilide (Ic)—To a magnetically stirred solution of freshly distilled 2,6-dimethylaniline (1.21 g, 0.01 mole) and *N*-benzyloxycarbonylglycine⁷ (2.10 g, 0.01 mole) in dichloromethane (50 ml) was added dicyclohexylcarbodiimide⁷ (25 ml) over 20 min. During the addition, a heavy white precipitate formed. Additional dichloromethane (75 ml) was added, and the slurry was stirred for 2 hr at room temperature.

The white precipitate, containing both dicyclohexylurea and ω -benzyloxycarbonylamino-2,6-dimethylacetanilide, was collected by suction

¹ IR spectra were recorded on either a Perkin-Elmer 337 or Varian IR-5A spectrophotometer. NMR spectra in chloroform- d_3 , unless otherwise indicated, were recorded on a Varian A-60 (δ) or T-60 (τ); all chemical shift values are reported as δ values with tetramethylsilane as internal standard. Electron-impact mass spectra were obtained on an AEI MS 902 (direct inlet; 70 eV); chemical-ionization mass spectra were obtained on an AEI MS-9 double-focusing mass spectrometer modified for chemical ionization, using isobutane as the reagent gas (17).

² Fischer-Johns.

³ Gold shield.

⁴ Huffman Laboratories, Wheatbridge, Colo.

⁵ Astra Pharmaceutical Products, Worcester, Mass.

⁶ Drierite.

⁷ Aldrich Chemicals, San Leandro, Calif.

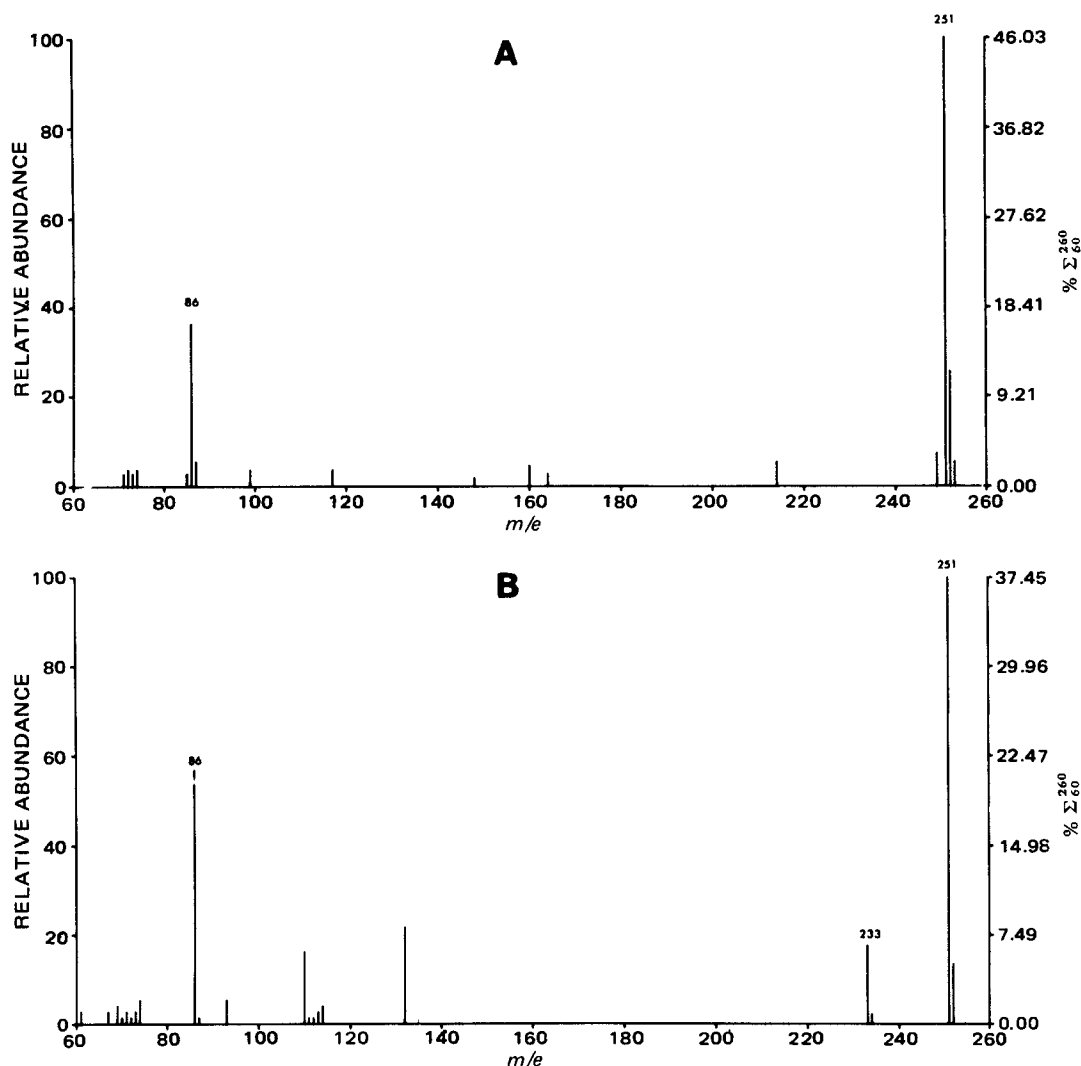


Figure 1—Comparative chemical-ionization mass spectra of Id (A) and If (B).

filtration and dissolved in aldehyde-free ethanol (75 ml) (21). The mixture was hydrogenated (25 psi hydrogen) on a Paar shaker over 300 mg of 10% palladium-on-carbon for 15 hr at room temperature. Rotary evaporation of ethanol gave a pale-yellow liquid, which was dissolved in purified chloroform (50 ml) (22) and extracted with two portions of 10% HCl (25 ml). Combined acid extracts were made basic with 5 *N* NaOH and back-extracted into ether.

The ethereal solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to yield a semisolid mass. Recrystallization from purified hexane-2-propanol gave 750 mg (42%) of white needles, mp 80–88° [lit. (15) mp 79–80°]; IR (KBr): 3400 (NH₂), 3300 (amide NH), and 1660 (amide C=O) cm⁻¹; NMR: δ^T 1.55 (broad m, NH₂), 2.20 (s, aryl methyls), 3.50 (t, *J* = 7 Hz, O=CCH₂NH₂), 7.05 (s, aromatics), and 8.80 (broad s, HNC=O) ppm. Addition of deuterium oxide showed loss of the broad multiplet at 1.65 ppm and collapse of the triplet at 3.50 ppm to a singlet.

The isobutane chemical-ionization mass spectrum of Ic showed that the MH⁺ ion at *m/e* 179 and its ¹³C-satellite represented 96.8% and *m/e* 164 was 3.2% of the total ion current. The electron-impact spectrum of Ic was published previously (15).

α -²H- ω -Amino-2,6-dimethylacetanilide (Ic-d₂)—To a solution of ω -amino-2,6-dimethylacetanilide (750 mg) in 99.7% deuterium oxide (20 ml) and freshly distilled tetrahydrofuran (5 ml) was added 0.5 g of anhydrous potassium carbonate. The solution was heated at 90° for 24 hr, cooled, and lyophilized; this procedure was repeated three times. After the final lyophilization, the white crystalline residue was taken up in purified chloroform (50 ml) and water (30 ml) and shaken vigorously. The chloroform extract was washed with two additional portions of water (10 ml) and dried over anhydrous sodium sulfate. Rotary evaporation yielded a pale-yellow crystalline solid, which was recrystallized from purified

hexane-2-propanol to give 510 mg (68%) of white needles, mp 79–80.5°; NMR: δ^T 1.55 (broad s, NH₂), 2.20 (s, aryl methyls), and 8.80 (broad s, HNC=O) ppm.

The chemical-ionization mass spectrum showed the following isotopic abundances: Ic-d₂, 99.0% (*m/e* 181); Ic-d₁, 1.0% (*m/e* 180); and Ic, 0% (*m/e* 179).

3-Hydroxy- ω -diethylamino-2,6-dimethylacetanilide (Id)—Compound Id was synthesized in 52% overall yield, according to the method of Keenaghan and Boyes (12), by nitration of lidocaine, reduction to the aromatic amino compound, diazotization, and hydrolysis to yield the phenol as white hexagonal plates from benzene-ethyl acetate, mp 203–204°. The chemical-ionization mass spectrum is shown in Fig. 1.

3-Hydroxy- ω -(α , α -²H-diethylamino)-2,6-dimethylacetanilide (Id-d₄)—Compound Id-d₄ was synthesized in 57% overall yield, according to the method of Keenaghan and Boyes (12), starting with lidocaine-d₄, mp 202–204°, after sublimation (140°, 0.3 mm Hg); NMR: δ 1.13 [broad s, N(CD₂CH₃)₂], 2.05 and 2.17 (s, aryl methyls), 3.30 (s, O=CCH₂N), 6.75 (AB m, *J* = 9 Hz, aromatics), and 8.90 (broad s, HNC=O) ppm.

The chemical-ionization mass spectrum showed the following isotopic abundances: Id-d₄, 71.0% (*m/e* 255); Id-d₃, 22.9% (*m/e* 254); Id-d₂, 4.0% (*m/e* 253); Id-d₁, 2.0% (*m/e* 252); and Id, 0% (*m/e* 251).

3-Hydroxy- ω -ethylamino-2,6-dimethylacetanilide (Ie)—Compound Ie was synthesized in 38% overall yield, according to the method of Keenaghan and Boyes (12), using the same reaction sequence as described for Id but starting with Ib, to yield off-white small plates from purified chloroform, mp 147–149°; NMR: δ^T 1.18 (t, *J* = 7 Hz, NCH₂CH₃), 2.05 and 2.17 (s, aryl methyls), 2.80 (q, *J* = 7 Hz, NCH₂CH₃), 3.40 (s, O=CCH₂N), 4.60 (broad s, OH and NH), 6.75 (AB m, *J* = 9 Hz, aromatics), and 8.90 (broad s, HNC=O) ppm.

3-Hydroxy- ω -(β -²H-ethylamino)-2,6-dimethylacetanilide (Ie-d₃)—Compound Ie-d₃ was synthesized in 28% overall yield, according to the method of Keenaghan and Boyes (12), starting with Ib-d₃, mp 147–149°; NMR: δ 2.05 and 2.17 (s, aryl methyls), 2.80 (broad s, NCH₂CD₃), 3.40 (s, O=CCH₂N), 4.60 (broad s, OH and NH), 6.75 (AB m, J = 9 Hz, aromatics), and 8.90 (broad s, HNC=O) ppm.

The chemical-ionization mass spectrum showed the following isotopic abundances: Ie-d₃, 64.8% (m/e 226); Ie-d₂, 22.5% (m/e 225); Ie-d₁, 9.0% (m/e 224); and Ie, 3.7% (m/e 223).

2-Amino-3-methylbenzyl Alcohol (Iib)—A solution of 2-amino-3-methylbenzoic acid⁷ (151 mg, 1 mmole) in tetrahydrofuran (10 ml, freshly distilled from lithium aluminum hydride) was added dropwise to a magnetically stirred slurry of lithium aluminum hydride (60 mg, 1.7 mmoles) in tetrahydrofuran (10 ml) over 20 min. The reaction was stirred 1 additional hr at room temperature. To the slurry, water (0.06 ml), 15% NaOH (0.06 ml), and water (0.18 ml) were added in succession. After stirring for 15 min, the granular white precipitate was removed by suction filtration. Rotary evaporation of the filtrate yielded 120 mg (89%) of off-white crystalline solid, which was recrystallized from chloroform-hexane (3:2) to give white prisms, mp 69–70° [lit. (23) mp 71°]; IR (KBr): 3450 (OH) and 3300 (NH₂) cm⁻¹; NMR: δ 2.05 (s, aryl methyls), 3.58 (broad s, NH₂ and OH), 4.41 (s, benzyl CH₂), and 6.80 (A₂B m, aromatics) ppm.

Chemical-ionization mass spectrometry showed that the MH⁺ ion at m/e 138 and its ¹³C-satellite represented 46.6% while (MH⁺ – 18) at m/e 120 was 53.4% of the total ion current.

2-Amino-3-methyl- α -²H-benzyl Alcohol (Iib-d₂)—Compound Iib-d₂ was synthesized in 92% yield, using the same procedure outlined for the nondeuterated analog with lithium aluminum deuteride, mp 69–70°; NMR (δ) showed the same peaks as the nondeuterated compound except for the loss of resonance at 4.41 ppm for the methylene hydrogens of the benzyl carbon.

Chemical-ionization mass spectrometry showed the following isotopic abundances: Iib-d₂, 91.8% (m/e 140); Iib-d₁, 8.2% (m/e 139); and Iib, 0% (m/e 138).

(²H-2-Methyl)-2,6-dimethylaniline (IIa-d₂)—A solution of Iib-d₂ (1.37 g, 10 mmoles) in 95% ethanol (50 ml) containing concentrated hydrochloric acid (1 ml) was hydrogenated (30 psi hydrogen) over 10% palladium-on-carbon (150 mg) for 4 hr on a Paar hydrogenator at room temperature. The catalyst was removed by suction filtration, and the filtrate was rotary evaporated to yield a semisolid residue. This residue was taken up in water (20 ml) and made basic with 10% Na₂CO₃, and the free amine was extracted into ether. The extract was washed with water and dried over calcium sulfate. The solvent was removed, and the product was vacuum distilled to give 950 mg (79%) of a clear liquid (bp 37–41°/0.3 mm Hg); NMR: δ 2.07 (s, aryl methyls), 3.42 (broad s, NH₂), and 6.8 (A₂B complex m, aromatics) ppm; integration ratio 4:2:3, respectively.

Chemical-ionization mass spectrometry showed the following isotopic abundances: IIa-d₂, 77.2% (m/e 124); and IIa-d₁, 22.8% (m/e 122).

ω -Diethylamino-2-hydroxymethyl-6-methylacetanilide (If)—To an ice bath-cooled, magnetically stirred solution of Iib (2.6 g, 20 mmoles) in tetrahydrofuran (30 ml) and benzene (20 ml) was added dropwise a solution of chloroacetic anhydride (3.4 g, 20 mmoles) in tetrahydrofuran (30 ml). After 30 min, a white crystalline precipitate formed and continued to form over the entire addition period. The slurry was warmed to room temperature and stirred an additional 2 hr. After cooling again in ice, the slurry was suction filtered, and the filter pad was washed with three portions of water (30 ml). After air drying, the white crystals were recrystallized from chloroform (40 ml) to yield 3.7 g (94%) of ω -chloro-2-hydroxymethyl-6-methylacetanilide, mp 130–132°; IR (KBr): 3400 (OH), 3300 (amide NH), and 1665 (amide C=O) cm⁻¹; NMR: δ 2.17 (s, aryl methyl), 2.77 (broad s, OH), 4.20 (s, O=CCH₂Cl), 4.58 (s, CH₂OH), 7.20 (s, aromatics), and 8.38 (broad s, HNC=O) ppm.

Anal.—Calc. for C₁₀H₁₂ClNO₂: C, 56.20; H, 5.66; N, 6.56. Found: C, 56.15; H, 5.61; N, 6.52.

A solution of ω -chloro-2-hydroxymethyl-6-methylacetanilide (641 mg, 3 mmoles) and 0.78 ml of diethylamine (550 mg, 7.5 mmoles) in dry dioxane (20 ml) was heated at 70–75° for 3 hr, during which a white precipitate of diethylamine hydrochloride formed. The mixture was cooled to 15°, suction filtered to remove the amine salt, and rotary evaporated to yield a pale-yellow liquid. The liquid was recrystallized from 30 ml of benzene-petroleum ether (80:20) to give cubic white crystals, mp 113–115°; IR (KBr): 3400 (OH), 3300 (amide NH), and 1660 (amide C=O) cm⁻¹; NMR: δ 1.17 [t, J = 7 Hz, N(CH₂CH₃)₂], 2.15 (s, aryl methyl), 2.85 [q, J = 7 Hz, N(CH₂CH₃)₂], 3.38 (s, O=CCH₂N), 3.71 (broad s, OH), 4.57 (s, CH₂OH), 7.21 (s, aromatics), and 8.60 (broad s, HNC=O) ppm.

Chemical-ionization mass spectrometry showed that the MH⁺ ion at

m/e 251 and its ¹³C-satellite represented 64.0%, (MH⁺ – 18) at m/e 233 represented 11.5%, m/e 132 was 13.0%, and from m/e 100 to 300 was 11.5% of the total ion current.

Anal.—Calc. for C₁₄H₂₂N₂O₂: C, 67.17; H, 8.86; N, 11.19. Found: C, 67.41; H, 8.90; N, 11.41.

ω -Ethylamino-(α -²H-2-hydroxymethyl)-6-methylacetanilide (Ig-d₂)—Compound Ig-d₂ was synthesized in 79% yield, using the same procedure as described for the diethylamino derivative with a threefold excess of ethylamine and Iib-d₂, mp 81–83° from chloroform; IR (KBr): 3400 (OH and NH), 3300 (amide NH), and 1660 (amide C=O) cm⁻¹; NMR: δ 1.13 (t, J = 7 Hz, NCH₂CH₃), 2.21 (s, aryl methyl), 2.68 (q, NCH₂CH₃), 3.38 (s, O=CCH₂N), 3.68 (broad s, OH), 7.20 (s, aromatics), and 8.60 (broad s, HNC=O) ppm; high-resolution electron-impact mass spectrometry: calc. for C₁₂H₁₆²H₂N₂O₂, 224.1494; found, 224.1492.

Chemical-ionization mass spectrometry showed that the MH⁺ ion at m/e 225 and its ¹³C-satellite represented 52.8%, (MH⁺ – 18) at m/e 207 was 38.1%, m/e 122 was 5.2%, and m/e 104 was 3.9% of the total ion current.

2,6-Dimethylphenylhydroxylamine (Iic)—To a vigorously stirred solution of 2,6-dimethylnitrobenzene⁷ (15.1 g, 0.1 mole) in absolute ethanol (30 ml) was added in one portion a solution of ammonium chloride (1 g) in water (15 ml) followed by small portions of zinc dust (94%, 15.0 g). The temperature of the reaction mixture was maintained between 65 and 70° by the portionwise addition of zinc dust until all had been added. The grayish-white slurry was then allowed to cool slowly to 55° with continued vigorous stirring. The warm mixture was suction filtered, and the filter pad was washed with anhydrous ether (30 ml). Rotary evaporation of the filtrate gave a pale-yellow precipitate, which was recrystallized twice from purified petroleum ether-benzene (1:1) to yield 12.2 g (88%) of white needles, mp 103–104° [lit. (24) mp 99°]; IR (KBr): 3300 (NH stretch), 3200 (OH), and 860 (NO stretch) cm⁻¹; NMR: δ 2.35 (s, aryl methyls), 6.50 (broad s, NH and OH), and 7.05 (s, aromatics) ppm.

Chemical-ionization mass spectrometry showed that the MH⁺ ion at m/e 138 and its ¹³C-satellite represented 23.0%, (MH⁺ – 16) at m/e 122 was 56.0%, m/e 243 was 18.0%, and m/e 259 was 3% of the total ion current. The ions at m/e 243 and 259 probably resulted from reaction to yield dimerized products in the gas phase.

Anal.—Calc. for C₈H₁₁NO: C, 70.04; H, 8.08; N, 10.21. Found: C, 69.95; H, 8.11; N, 10.04.

ω -Chloro- N -hydroxy-2,6-dimethylacetanilide—To a magnetically stirred two-phase mixture of Iic (4.12 g, 0.03 mole) in ether (100 ml) and sodium bicarbonate (2.80 g, 0.034 mole) in water (12 ml), cooled to –5° in an ice-salt bath, was added dropwise chloroacetyl chloride⁷ (3.39 g, 0.03 mole) in ether (20 ml) over 30 min. The reaction was stirred an additional 20 min at –5° and then poured into a separator and washed with two portions of water (50 ml). The pale-yellow ether layer was filtered through anhydrous magnesium sulfate and evaporated to a volume of 50 ml, and the product was crystallized in the cold to yield 3.52 g (63%) of white rhombic crystals, mp 138.5–140°; IR (KBr): 3200 (OH) and 1650 (CO) cm⁻¹; NMR: δ 2.1 and 2.32 (s, aryl methyls), 3.76 and 4.41 (s, O=CCH₂Cl), 7.05 and 7.25 (m, aromatics), and 1.15 (broad s, deuterium oxide exchangeable OH) ppm. Integration indicated two isomers existing in a 1:2 ratio, presumably imino-enol to N -hydroxyamide.

Anal.—Calc. for C₁₀H₁₂ClNO₂: C, 56.21; H, 5.66; N, 6.56. Found: C, 56.33; H, 5.60; N, 6.51.

ω -Diethylamino- N -hydroxy-2,6-dimethylacetanilide (Ih)—A solution of ω -chloro- N -hydroxy-2,6-dimethylacetanilide (2.13 g, 0.01 mole) and diethylamine (2.8 ml = 2.2 g, 0.03 mole) in benzene (30 ml) was magnetically stirred for 4 hr at 65–70°. The orange-colored solution was cooled in ice, suction filtered to remove precipitated diethylamine hydrochloride, and then extracted with three portions of 10% HCl (5 ml). The acid extracts were combined and carefully adjusted to pH 8.5 with 5 *N* NaOH. The opaque mixture was back-extracted with three portions (20 ml) of purified chloroform, which were combined and washed with water (10 ml) and dried over anhydrous sodium sulfate.

Rotary evaporation of the solvent at room temperature yielded a pale-yellow liquid, which was recrystallized three times from purified hexane to yield 1.6 g (64%) of small white needles, mp 81–82°; IR (CCl₄): 3250 (OH) and 1650 (CO) cm⁻¹; NMR: δ 0.85 and 1.15 [t, J = 7 Hz, N(CH₂CH₃)₂], 2.30 and 2.35 (s, aryl methyls), 2.50–3.00 [overlapped q, J = 7 Hz, N(CH₂CH₃)₂], 3.55 and 4.60 (s, O=CCH₂N), and 7.10–7.20 (m, aromatics plus OH exchangeable with deuterium oxide) ppm. Integration indicated two isomers in a ratio of 1:1; high-resolution electron-impact mass spectrometry: calc. for C₁₄H₂₂N₂O₂, 250.16812; found, 250.16873.

Chemical-ionization mass spectrometry showed that the MH⁺ ion at m/e 251 and its ¹³C-satellite represented 65.1%, (MH⁺ – 16) at m/e 235

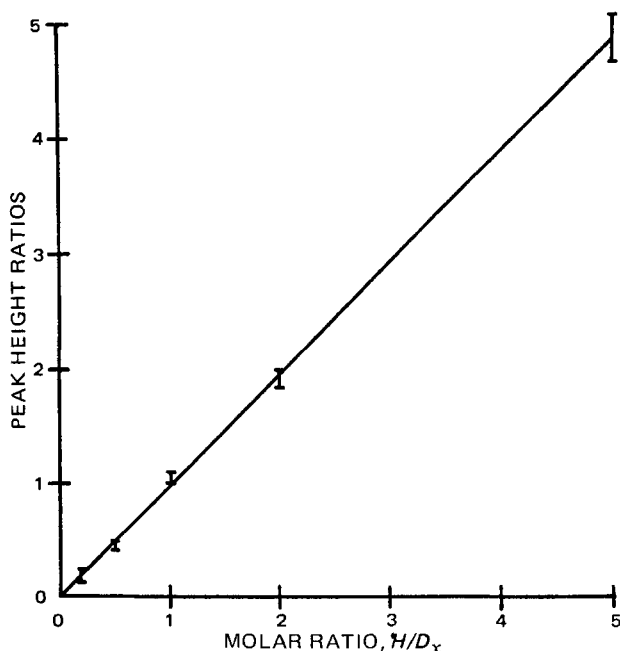


Figure 2—Standard curve of lidocaine and six of its metabolites plus their deuterated standards extracted from urine. The bars (I) represent the range of peak height ratios observed in chemical-ionization mass spectral analysis for various amounts of lidocaine and the six metabolites added to 10-ml blank urine samples containing the following amounts of deuterated standards: 100 μ g of lidocaine- d_4 , 150 μ g of Ib- d_3 , 100 μ g of Ic- d_2 , 50 μ g of Id- d_4 , 50 μ g of Ie- d_3 , 50 μ g of IIa- d_2 , and 200 μ g of IId- d_2 . The variances observed for all compounds and their deuterated analogs were from 1 to 5%, except for samples of Ic versus Ic- d_2 where variances as much as $\pm 10\%$ were found due to interfering ions observed in the chemical-ionization mass spectra of control urine at m/e 179, 180, and 181.

was 19.9%, m/e 233 was 11.7%, and m/e 103 from m/e 100 to 300 was 3.3% of the total ion current.

Anal.—Calc. for $C_{14}H_{22}N_2O_2$: C, 67.17; H, 8.86; N, 11.19. Found: C, 67.40; H, 8.70; N, 11.30.

The hydrochloride was made by adding the calculated amount of concentrated hydrochloric acid to a solution of Ih in 2-propanol and cooling the solution in a freezer to give small white crystals, mp 182–184°; IR (KBr): 3450 (OH), 2700 (N^+H), and 1665 ($C=O$) cm^{-1} ; NMR: δ (acetone- d_6 , tetramethylsilane) 1.45 [t, $J = 7$ Hz, $N(CH_2CH_3)_2$], 2.30 (s, aryl methyls), 3.55 [q, $J = 7$ Hz, $N(CH_2CH_3)_2$], 4.09 (broad s, N^+H and OH), 4.72 (s, $O=CCH_2N$), and 7.23 (s, aromatics) ppm.

ω -(α,α - 2H -Diethylamino)- N -hydroxy-2,6-dimethylacetanilide (Ih- d_4)—Compound Ih- d_4 was synthesized by the same procedure as the nondeuterated compound, using diethylamine- d_4 , mp (hydrochloride) 182–184°.

Chemical-ionization mass spectrometry showed the following isotopic abundances: Ih- d_4 , 72.0% (m/e 255); Ih- d_3 , 20.6% (m/e 254); Ih- d_2 , 5.9% (m/e 253); Ih- d_1 , 1.5% (m/e 252); and Ih, 0% (m/e 251).

ω -Ethylamino- N -hydroxy-2,6-dimethylacetanilide (II)—A solution of ω -chloro- N -hydroxy-2,6-dimethylacetanilide (2.13 g, 0.01 mole) and anhydrous ethylamine (1.95 ml = 1.35 g, 0.03 mole) in purified dioxane (30 ml) was stirred for 4.5 hr at 50–55°. As the reaction proceeded, a large amount of fine white precipitate formed. The slurry was cooled in ice and suction filtered to give a mixture of product and ethylamine hydrochloride (2.3 g, mp 70–102°). The precipitate was taken up in chloroform-petroleum ether (2:1) and suction filtered to remove the ethylamine hydrochloride. The filtrate was then evaporated, and the residue was recrystallized twice from benzene to yield 1.4 g (63%), mp 103–105°; IR (CCl₄): 3250 (OH) and 1650 ($C=O$) cm^{-1} ; NMR: δ 1.05 and 1.17 (t, $J = 7$ Hz, NCH_2CH_3), 2.15 and 2.20 (s, aryl methyls), 2.57 and 2.64 (q, $J = 7$ Hz, NCH_2CH_3), 3.00 and 3.85 (s, $O=CCH_2N$), 5.95 (s, OH and NH exchangeable with deuterium oxide), and 7.10 and 7.20 (s, aromatics) ppm. Integration indicated two isomers in an approximate ratio of 1:2, with the presumed N -hydroxy form predominant; high-resolution electron-impact mass spectrometry: calc. for $C_{12}H_{18}N_2O_2$, 222.13726; found, 222.13682.

Chemical-ionization mass spectrometry showed that the MH^+ ion at

m/e 233 and its ^{13}C -satellite represented 70.4%, ($MH^+ - 16$) at m/e 207 was 20.8%, and ($MH^+ - H_2O$) at m/e 205 from m/e 100 to 300 was 8.8% of the total ion current.

Anal.—Calc. for $C_{12}H_{18}N_2O_2$: C, 64.84; H, 8.16; N, 12.60. Found: C, 64.75; H, 8.41; N, 12.62.

The hydrochloride was made by adding the calculated amount of concentrated hydrochloric acid to a solution of II in 2-propanol and cooling the solution in a freezer to give small white crystals, mp 186–188°; IR (KBr): 3450 (OH), 2700–2900 (N^+H), and 1665 ($C=O$) cm^{-1} ; NMR: δ (acetone- d_6 , tetramethylsilane) 1.40 (t, $J = 7$ Hz, NCH_2CH_3), 2.35 (s, aryl methyls), 3.35 (q, $J = 7$ Hz, NCH_2CH_3), 4.25 (broad s, N^+H and OH), 4.50 (s, $O=CCH_2N$), and 7.20 (s, aromatics) ppm.

ω -(β - 2H -Ethylamino)- N -hydroxy-2,6-dimethylacetanilide (II- d_3)—Compound II- d_3 was synthesized by the same procedure as the nondeuterated compound using ethylamine- d_3 , mp (hydrochloride) 185–187°.

Chemical-ionization mass spectrometry showed the following isotopic abundances: II- d_3 , 66.7% (m/e 226); II- d_2 , 19.3% (m/e 225); II- d_1 , 10.7% (m/e 224); and II, 3.3% (m/e 223).

N' -Ethyl-2-methyl- N^3 -(2,6-dimethylphenyl)-4-imidazolidinone (III)—Compound III was synthesized as previously described (25).

Lidocaine N -Oxide (Ij)—To an ice bath-cooled, magnetically stirred solution of lidocaine (2.34 g, 0.01 mole) in dichloromethane (20 ml) was added a cold solution of m -chloroperoxybenzoic acid⁷ (85%, 2.23 g, 0.011 mole) in dichloromethane (30 ml). After a dropwise addition over 30 min, the reaction mixture was allowed to come to room temperature and was stirred overnight. The reaction mixture was then poured through a basic alumina column (150 g). Unreacted lidocaine was eluted with chloroform (100 ml), followed by 200 ml of chloroform-methanol (95:5), which yielded a pale-yellow liquid after rotary evaporation of the solvent. Recrystallization from ether-dichloromethane (70:30) in the cold gave 1.8 g (72%) of a white hygroscopic crystalline solid, mp 105–107°; IR (KBr): 3100–3400 (OH of hydrate and amide NH) and 1690 (amide $C=O$) cm^{-1} ; NMR: δ 1.37 [t, $J = 7$ Hz, $N(CH_2CH_3)_2$], 2.25 (s, aryl methyls), 2.92 (broad s, $HNC=O$ and H_2O), 3.45 [q, $J = 7$ Hz, $N(CH_2CH_3)_2$], 3.90 (s, $O=CCH_2N$), and 7.06 (s, aromatics) ppm.

Chemical-ionization mass spectrometry showed that the MH^+ ion at m/e 251 and its ^{13}C -satellite represented 3.2%, ($MH^+ - 16$) at m/e 235 was 11.4%, ($MH^+ - H_2O$) at m/e 233 was 16.9%, m/e 223 was 19.4%, m/e 221 was 4.7%, m/e 207 was 8.4%, m/e 205 was 17.9%, and m/e 178 from m/e 100 to 300 was 11.9% of the total ion current.

Anal.—Calc. for $C_{14}H_{22}N_2O_2 \cdot H_2O$: C, 62.66; H, 9.01; N, 10.44. Found: C, 62.37; H, 8.81; N, 10.43.

4-Hydroxy-2,6-dimethylaniline (IId)—Compound IId was synthesized according to known procedures (26), mp 179–181°.

4-Hydroxy-(2H -2-methyl)-2,6-dimethylaniline (IId- d_2)—Compound IId- d_2 was synthesized by the method outlined by Wepster (27). The method involved formation of the p -toluenesulfonamide of IIa- d_2 , followed by nitration and reduction to yield the p -amino- p -toluenesulfonamide. This material was diazotized, treated with water to yield the phenol, and finally hydrolyzed to yield crude IId- d_2 . The crude material was sublimed (80–90°, 3 mm Hg) to give 60% very pure white powder crystals, mp 179–181°, and 40% slightly impure light-purple crystals, mp 175–180°; NMR: δ 2.06 (s, aryl methyls), 4.22 (broad s, NH_2), 6.47 (s, OH), and 6.59 (s, aromatics) ppm; integration ratio 4:2:1:2, respectively; high-resolution electron-impact mass spectrometry: calc. for $C_8H_9^2H_2NO$, 139.0966; found, 139.0932.

Chemical-ionization mass spectrometry showed that MH^+ ions represented 100% of the total ion current with the following isotopic abundances: IId- d_2 , 60.2% (m/e 140); IId- d_1 , 22.4% (m/e 139); and IId, 17.4% (m/e 138).

N,N -Diethylglycine (IVa)—Compound IVa was prepared by the method of Bowman and Stroud (28), using reductive alkylation of glycine with excess acetaldehyde and hydrogen, mp 133–135°.

N,N - α,α,α' - 2H -Diethylglycine (IVa- d_4)—A mixture of glycine (3.8 g, 0.05 mole) and freshly distilled acetaldehyde (9.0 g, 0.2 mole) in 70% ethanol- d_1 -deuterium oxide was hydrogenated (20 psi, deuterium) over 1.5 g of 10% palladium-on-carbon for 14 hr at room temperature. The catalyst was removed by suction filtration, and the filtrate was rotary evaporated to yield a viscous pale-yellow liquid, which was recrystallized as the hydrochloride from 75 ml of methanol-acetone (1:4) to give 5.7 g (67%) of a white crystalline solid, mp 124–126° [lit. (29) mp 126–127°].

Chemical-ionization mass spectrometry showed that deuterium scrambling had occurred during the reductive alkylation, with incorporation ranging from zero to eight deuteriums into the N -alkyl side chains. The resultant deuterium envelope showed the greatest deuterium incorporation for IVa- d_3 , 21.0% (m/e 135). Parent $MH(D)^+$ ions repre-

Table I—Serial Plasma Levels of Lidocaine, Ib, and III

Minutes ^a	Subject	Lidocaine		Ib		III	
		<i>m/e</i> 235 ^b	$\mu\text{g/ml}$	<i>m/e</i> 207 ^b	$\mu\text{g/ml}$	<i>m/e</i> 233	$\mu\text{g/ml}$
		<i>m/e</i> 239		<i>m/e</i> 210		<i>m/e</i> 235	
30	A	84.2/29.6	2.03	22.3/90.0	0.13	0.049	0.10
	B	93.5/83.3	0.81	10.2/38.7	0.13	0.043	0.03
	C	18.1/90.6 ^c	0.002	Not measurable		Not measurable	
60	A	90.6/44.0	1.47	45.2/41.0	0.55	0.075	0.11
	B	60.3/64.8	0.67	17.4/34.5	0.25	0.079	0.05
	C	5.3/64.2	0.05	5.8/50.3	0.06	0.093	0.04
90	A	60.5/34.9	1.22	11.9/26.6	0.23	0.048	0.06
	B	50.7/77.8	0.47	21.6/34.5	0.21	0.054	0.02
	C	40.7/68.8	0.42	7.3/57.7	0.06	0.076	0.04
180	A	49.0/37.0	0.95	8.7/19.7	0.22	0.066	0.06
	B	38.7/121	0.23	10.1/26.6	0.16	0.063	0.01
	C	58.7/102	0.41	7.9/60.8	0.06	0.112	0.04

^a Refers to the time the blood sample was taken after subject received an oral dose of lidocaine hydrochloride monohydrate (A and B) or lidocaine as the free base (C).
^b The peak heights as measured from the chemical-ionization mass spectrum after correcting for ¹³C-abundances and deuterium incorporation. ^c Based on ¹³C-satellite peak of lidocaine-*d*₄ internal standard.

sented 70.1% and (MH⁺ - C₂H₂O₂) ions were 22.4% of the total ion current.

N-Ethylglycine (IVb)—Compound IVb was prepared by the method of Hanke (30), which involves hydrolysis of *N*-ethylaminoacetonitrile generated from the condensation of ethylamine, formaldehyde, and potassium cyanide.

β -²H-*N*-Ethylglycine (IVb-*d*₃)—Compound IVb-*d*₃ was synthesized by the method of Hanke (30), using ethylamine-*d*₃ hydrochloride, mp 180–182° dec.

Chemical-ionization mass spectrometry showed the following isotopic abundances: IVb-*d*₃, 57.9% (*m/e* 107); IVb-*d*₂, 20.8% (*m/e* 106); IVb-*d*₁, 13.2% (*m/e* 105); and IVb, 8.1% (*m/e* 104).

Mass Spectral Studies—Chemical-ionization mass spectrometry was performed on all suspected lidocaine metabolites and their deuterated analogs. All of the benzyl alcohols (If, Ig, and Ib) produced major (MH⁺ - 18) ions as a result of a loss of water from the MH⁺ ion. Because of this characteristic water loss, any benzyl alcohol metabolites should be easily differentiated from the isomeric phenolic metabolites (Id, Ie, and IId). Figure 1 shows comparative chemical-ionization mass spectra of If and Id.

Unfortunately, the *N*-hydroxyamides (Ih and Ii), IIc, and Ij lost oxygen under all temperature and chemical-ionization mass spectrometry conditions employed to give (MH⁺ - 16) ions, which interfered with quantification of lidocaine, Ib, and 2,6-dimethylaniline. Therefore, these possible oxygenated metabolites were quantified by a combination of the chemical-ionization mass spectrometry-stable isotope technique and other methods as previously described (31). This facile loss of oxygen is probably the result of a thermal homolytic cleavage of the weak nitrogen-oxygen bond and was observed previously (32, 33).

Preliminary Experiment—To determine the spectrum of lidocaine metabolites detectable by chemical-ionization mass spectrometry in plasma and urine samples, a healthy 75-kg male subject received orally an encapsulated dose of 202 mg of lidocaine free base. Prior to the dose, a 24-hr urine control sample was collected and stored in a freezer. Immediately prior to dosing, a 15-ml "zero-time" blood sample was collected in a heparinized tube, and the plasma was separated by spinning the sample at 800×g for 15 min. Similar samples were collected at 30, 60, 90, and 180 min after dosing and stored in a freezer at -15° until further workup. Serial urine samples were collected at the following intervals: 0–2, 2–4, 4–8, and 8–24 hr, and stored in glass bottles in a freezer.

Plasma and urine samples were extracted at pH 8.5 with two volumes of dichloromethane. The residue, after evaporation of the solvent, was subjected to chemical-ionization mass spectrometry. Compared to the zero-time plasma sample, only MH⁺ ions corresponding to lidocaine, Ib, and possibly Ic could be seen in the plasma extracts. In the urine extracts, MH⁺ ions corresponding to IIa, IId, Ic, Ib, hydroxylated Ib, Ia, and hydroxylated Ia could readily be detected. No major peaks corresponding to the (MH⁺ - 18) loss of water from possible benzyl alcohol metabolites could be found, even though these compounds were readily extractable from urine and plasma.

Isolation and Assay Procedures—Collection bottles for urine and Pyrex culture tubes for plasma were thoroughly washed with steaming water and detergent, rinsed three times with hot water, and finally rinsed five times with distilled water. A stock solution (200 ml) containing 1.60 mg of Id-*d*₄, 8.00 mg of Ia-*d*₄, 1.60 mg of Ie-*d*₃, 20.00 mg of Ib-*d*₃, 4.00 mg of Ic-*d*₂, and 2.06 mg of IIa-*d*₂ was made the morning of the *in vivo* ex-

periment. All weighings were performed on an electrobalance⁸. All solvents used for extractions were purified (23) and distilled prior to use. Conjugates were hydrolyzed enzymatically⁹.

Two healthy male volunteers (A = 80 kg, B = 70 kg) received a 250-mg oral dose of encapsulated lidocaine hydrochloride monohydrate¹⁰; a third subject (C = 75 kg) received a molar equivalent (202 mg) of lidocaine free base¹¹. All doses were given in the morning with no restriction on diet or smoking. Prior to the experiment, 24-hr urine controls were collected from each subject and stored in a freezer (-15°). Immediately prior to dosing, a 15-ml zero-time blood sample was drawn into a heparinized tube. Successive 15-ml blood samples were taken 30, 60, 90, and 180 min following drug administration; the plasma was immediately separated by centrifugation at 800×g for 15 min. To 2-ml samples of each plasma fraction were added 2 ml of 1.01 μg of Ia-*d*₄/ml, 1 ml of 1.00 μg of Ib-*d*₃/ml, and 0.5 ml of 1.00 μg of Ic-*d*₂/ml. Samples were then stored frozen until further workup.

Serial urine samples were collected 0–2, 2–4, 4–8, and 8–24 hr following drug administration. One-third of each sample was transferred at the collection time to a glass bottle containing 10 ml of the stock solution of deuterated standards. To two-tenths of this sample containing the deuterated standards (times one-third) was added 1.0 mg of IId-*d*₂, and the samples were stored in a freezer (-15°) until further workup. To one-twentieth of the 0–24-hr urine aliquots from each subject were added 1.0 mg each of the deuterated amino acids, IVa-*d*₂ and IVb-*d*₃.

Plasma—All plasma samples were extracted the day following collection. To the 5.5 ml of sample containing 2 ml of plasma and 3.5 ml of deuterated standards solution was added 0.5 ml of 5 *N* NaOH, and the basified mixture was extracted with 10 ml of benzene in 30-ml Pyrex culture tubes fitted with polytet caps. The sample was then centrifuged at 800×g for 5 min, and the benzene layer was removed and rotary evaporated at 20°. The residue was dissolved in 0.05 ml of ethanol and placed on the ceramic tip of the direct insertion probe. The probe was inserted into the mass spectrometer, and the chemical-ionization mass spectrum was determined at 120°.

Urine—Urine samples, including urine controls, were extracted within 2 days following collection. One-half of each urine sample, containing all deuterated standards (i.e., $\frac{1}{2} \times \frac{1}{10} \times \frac{1}{2} = \frac{1}{40}$ of each serial urine sample), was basified to pH 8.5 ± 0.2 with 5 *N* NaOH and extracted with two volumes of purified dichloromethane. The extract was filtered through anhydrous sodium sulfate and rotary evaporated at 20°. The residue was transferred to a 1-dram vial with 1 ml of purified dichloromethane. The solvent was evaporated under a gentle stream of nitrogen, and samples were stored in a desiccator in a freezer until chemical-ionization mass spectrometry was performed.

The other half of each urine sample was diluted with an equal volume of pH 5.0 Walpole acetate buffer and hydrolyzed for 24 hr on a shaker-incubator set at 37.0° and 100 oscillations/min. β -Glucuronidase-sulfatase was added in two 0.5-ml portions, once at the beginning of the incubation and again 12 hr after the incubation was started. Extraction of each β -glucuronidase-treated sample was carried out in the same manner as for untreated urine. All of these samples, however, required a primary

⁸ Model M-10, Cahn electrobalance.

⁹ Sigma Type II β -glucuronidase-sulfatase from *Helix pomatia*.

¹⁰ Xylacaine, Astra Pharmaceutical Products, Worcester, Mass.

¹¹ Prepared in this laboratory.

Table II—Quantification of Lidocaine and Its Metabolites in Human Urine

Compound	Percentage of Administered Dose Recovered in 0–24-hr Urine ^a					
	Subject A		Subject B		Subject C	
	Before Enzyme, %	After ^b Enzyme, %	Before Enzyme, %	After ^b Enzyme, %	Before Enzyme, %	After Enzyme, %
Lidocaine	1.78	1.51	3.33	2.90	0.74	0.74
Ib	4.17	4.02	8.80	8.36	1.74	1.73
Ic	0.23	— ^c	1.32	— ^c	1.08	— ^c
<i>m</i> - and/or <i>p</i> -Hydroxylidocaine	0.06	0.80	0.10	0.43	0.06	0.97
<i>m</i> - and/or <i>p</i> -Hydroxy Ib	0	0.62	0.20	0.80	0.03	0.25
IIa	1.41	— ^c	0.27	— ^c	1.22	— ^c
IIc	1.37	68.2	0.70	61.7	0.	60.5
Total	9.02	75.2	14.7	74.2	4.87	64.2

^a Quantified as molar concentrations of free bases in Subjects A and B after an oral dose of 250 mg of lidocaine hydrochloride monohydrate and in Subject C after an oral dose of lidocaine free base. ^b After treatment with β -glucuronidase-sulfatase for 24 hr, some of the metabolite levels decreased inexplicably. ^c The β -glucuronidase-sulfatase treatment somehow affected both the metabolite and internal standard so that they were inextractable.

suction filtration through glass wool to break difficult emulsions before filtration through anhydrous sodium sulfate. All residues of the hydrolyzed urine were dark, foul smelling, and tarry.

Chemical-ionization mass spectrometric analysis was accomplished by dissolving each residue in 0.05 ml of methanolic hydrogen chloride, evaporating the sample on the probe tip with a gentle stream of nitrogen, and temperature programming each sample from 90 to 220°; spectra were taken approximately every 20°. Temperature programming was necessary to determine the more volatile IIa (90–110°) and IIc (120–150°) and also to rid the spectrum of some interfering peaks near *m/e* 180 so that Ic could be determined above 170°. The methanolic hydrogen chloride treatment served two purposes. It converted the metabolites to their less volatile amine hydrochlorides and, combined with temperature programming, it destroyed any *N*-hydroxyamides that might have been present by causing Bamberger-type reactions (34) so that these compounds would not interfere with quantification of lidocaine and Ib.

Calculations—Peak heights were measured using a caliper. A standard procedure (35) was followed in subtracting isotope contributions from the preceding two peaks. The contributions of nondeuterated or unreacted ionic species present in the reference solution (internal standard) were then subtracted. A comparison of the corrected peak heights of labeled and unlabeled compound was made, and the concentration of drug or metabolite initially present in the plasma or urine sample was determined.

Standardization Experiments—*Plasma*—Previous work showed that extraction and mass spectral response to a compound and a deuterated analog of that compound are equivalent (36, 37). To test this finding under the chemical-ionization mass spectrometry conditions used, Ia and Ia-*d*₄ (1 μ g/ml), Ib and Ib-*d*₃ (0.5 μ g/ml), and Ic and Ic-*d*₂ (0.25 μ g/ml) were added to 2-ml blank plasma samples and extracted according to the procedures outlined previously. Chemical-ionization mass spectrometric analysis was performed on duplicate samples and the corrected peak height ratios of nondeuterated *versus* deuterated standards showed good agreement between known and calculated concentrations, the largest variance being 2.2% for Ia *versus* Ia-*d*₄.

Urine—To 10 10-ml blank urine samples in 50-ml Pyrex culture tubes was added 1.0 mg of IIc-*d*₂ plus 1 ml of the deuterated standards stock solution. To these samples was added 1 ml of stock solutions containing the same nondeuterated compounds to obtain five sets of duplicate samples with concentrations of nondeuterated-deuterated analogs ranging from 1:5 to 5:1. The 12 ml of solution was basified to pH 8.5 with 5 *N* NaOH and extracted with 20 ml of purified dichloromethane. Chemical-ionization mass spectrometric analysis with temperature programming was performed as described previously. Calculations showed that the response was linear over the range analyzed and that the lidocaine-derived compounds and their deuterated analogs behaved identically (Fig. 2).

Quantification of Amino Acid Metabolites—All radioactive determinations (*MSE* = 1.5%) were made using scintillation counting¹². The samples were dissolved in 10 ml of scintillation fluid¹³ and corrected for quenching using internal standardization with either ³H-toluene standard¹³ or ¹⁴C-toluene standard¹³. GLC was performed¹⁴ using

flame-ionization detectors with a nitrogen carrier gas flow rate at 40 ml/min. Separation was obtained at 55° on 3% OV-17 on 100–120-mesh Chromosorb W in a 1.67-m $\times$ 0.63-cm (5.5-ft $\times$ 0.25-in.) $\times$ 2-mm i.d. glass U column equipped with a 10:1 effluent splitter.

Urease¹⁵ was used to hydrolyze urea. TLC separation was accomplished on 20 $\times$ 20-cm plates¹⁶ with silica gel or cellulose adsorbent.

Using Radioactivity Measurements—Two human subjects received an oral dose of 500 mg of randomly tritiated lidocaine hydrochloride monohydrate (13). An aliquot of the urine (0–72 hr) was treated with urease and, after filtration, was lyophilized. The lyophilate was extracted (soxhlet) with successive portions of dichloromethane, 2-propanol, and methanol to constant activity. The 2-propanol extract, accounting for 70% of the activity in the lyophilate, was evaporated under reduced pressure, taken up in 10 ml of water, and placed on a 250-ml (20 $\times$ 4-cm) cation-exchange column¹⁷, which was then washed with 1.5 liters of water, 2 liters of 8 *N* acetic acid, and 4 liters of water. Very small amounts of activity were eluted in these fractions. Finally, a linear gradient from water to 2 *N* HCl was run (2 liters total volume), and 10-ml fractions were collected.

Eighty percent of the activity applied to the column was found between fractions 105 and 130. The fractions were combined and lyophilized repeatedly to remove excess hydrochloric acid, and the white residue was washed repeatedly with 2-propanol. Combined extracts were evaporated to a small volume and streaked on two 20 $\times$ 20-cm silica gel plates¹⁸. The plates were developed twice in butanol-acetic acid-water (4:1:1). Radiochromatogram scanning showed only one radioactive band, *R_f* 0.3, which was scraped from the plates and eluted with ethanol. Evaporation of ethanol yielded a residue, which was treated with diazomethane in ether and gas chromatographed. One peak was found (retention time of 6.2 min), collected, and determined to be radioactive by scintillation counting.

Electron-impact mass spectrometry of the trifluoroacetic acid salt of the methylated amino acid showed peaks at *m/e* 145, 86, 69, and 58. *N,N*-Diethylglycine, prepared by the method of Bowman and Stroud (28) and methylated with thionyl chloride in methanol, showed identical GLC and mass spectral characteristics. Isotopic dilution of samples of the isolated amino acid in the synthetic amino acid, with continuous recrystallization to constant specific activity, confirmed the identification of the metabolite.

Aliquots (100 ml) of urine from two subjects receiving 500 mg of randomly tritiated lidocaine hydrochloride monohydrate (13) were incubated with urease and filtered, and 50 ml of the filtrate was lyophilized with 362 mg of nonradioactive synthetic *N,N*-diethylglycine. The material was lyophilized twice to ensure removal of any exchangeable tritium. The lyophilate was triturated with three 50-ml portions of boiling benzene and filtered, and the benzene was evaporated carefully under nitrogen at room temperature. The residue was recrystallized repeatedly from boiling benzene to constant specific activity.

Using Chemical-Ionization Mass Spectrometry—Stable Isotope Dilution Technique—The one-twentieth aliquots of urine from each subject, containing 1.0 mg each of the deuterated amino acids, were

¹² Tri-Carb model 3375 scintillation counter.

¹³ Aquasol, New England Nuclear, Boston, Mass.

¹⁴ Model 1100, Varian Aerograph.

¹⁵ Sigma Type M.

¹⁶ Eastman Chemical.

¹⁷ Dowex AG-50 WX4, hydrogen form.

¹⁸ Analtech, silica gel HR, 2504.

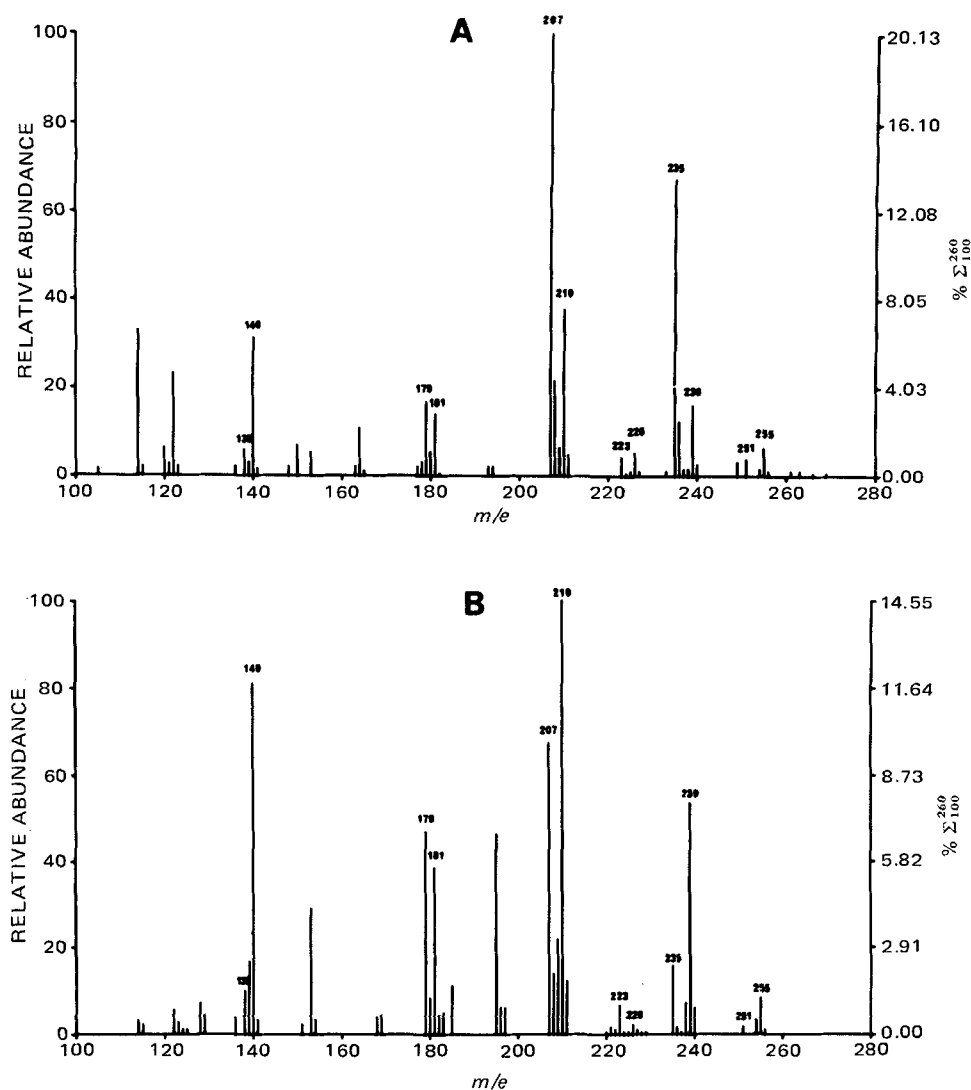


Figure 3—Comparative chemical-ionization mass spectra of the methylene chloride extracts from basified (pH 8.5) Subject B 0-2-hr (A) and 8-24-hr (B) urine samples. The labeled peaks represent the following metabolites and their deuterated standards: *m/e* 138, II_d; *m/e* 140, II_d-d₂; *m/e* 179, I_c; *m/e* 181, I_c-d₂; *m/e* 207, I_b; *m/e* 210, I_b-d₃; *m/e* 223, *m*- and/or *p*-I_e; *m/e* 226, I_e-d₃; *m/e* 235, I_a; *m/e* 239, I_a-d₄; *m/e* 251, *m*- or *p*-I_d; and *m/e* 255, I_d-d₄.

treated with urease (100 mg) at 30° with shaking for 72 hr in an equal volume of pH 7.0 sodium phosphate buffer. Each sample was then lyophilized to yield approximately 600 mg of light-brown powder, which was extracted with 10 ml of hot methanol. The methanol extract was rotary evaporated, and the residue was sublimed (130°/0.3 mm Hg) for 3 hr. The sublimate was taken up in a few drops of methanol and chromatographed on silica gel plates, using 1-butanol-water-acetic acid (8:3:2) as the developing solvent. The appropriate area (*R_f* 0.2-0.4), as determined by cochromatography with standards, was scraped off and eluted with methanol, followed by rechromatography in the same system. TLC on silica gel of a sample of the final extract showed a ninhydrin-sensitive area corresponding to the *R_f* region for both *N,N*-diethylglycine and monoethylglycine. TLC on cellulose, using 95% ethanol-2-propanol-water (6:3:1), also indicated ninhydrin-sensitive areas for *N,N*-diethylglycine (*R_f* 0.51, very pale-pink spot) and monoethylglycine (*R_f* 0.27, purple-pink spot).

Chemical-ionization mass spectrometry of each extract showed small peaks at both *m/e* 104 and 132 corresponding to the amino acids, along with envelopes of peaks attributable to their deuterated internal standards. However, due to the large number of peaks in the same mass range, meaningful quantification was virtually impossible.

RESULTS

Plasma—The results of the quantification are presented in Table I and were partially given in a previous publication (16). In all samples with

measurable levels of I_b, small but measurable amounts of what appeared to be Metabolite III could be detected. Based on the peak height ratio of the MH⁺ of the cyclic metabolite at *m/e* 233 versus the MH⁺ ion of lidocaine at *m/e* 235, the concentration of the cyclic metabolite in plasma followed approximately the same time course as I_b¹⁹. The peak concentration appeared at 60 min and reached a concentration level of approximately one-fifth that of the I_b level in Subjects A and B (Table I). These concentrations of cyclic metabolite must be considered as rather crude estimates. However, the presence of this metabolite as a real *in vivo* generated product is strongly suggested, since there was no concomitant increase in *m/e* 236 compared to control values. This finding would be expected if the substance was arising from the condensation of exogenous acetaldehyde with I_b (25), since the acetaldehyde should also condense with the deuterated analog to generate *m/e* 236. The problem of the cyclic metabolite will be considered in a future publication.

Urine—Lidocaine and six of its metabolites were quantified simultaneously using the chemical-ionization mass spectrometry-stable isotope dilution procedure. Basically, this procedure involved collection of serial urine samples with immediate addition of a stock solution containing known amounts of the deuterated standards to urine aliquots. The urine

¹⁹ Although the MH⁺ ion for the cyclic metabolite was found in all plasma samples, the deuterium standard of the cyclic metabolite was not added to the plasma because of its facile hydrolysis to I_b plus acetaldehyde. Due to the almost identical extraction characteristics of the cyclic metabolite and lidocaine, deuterated lidocaine was used as the reference standard.

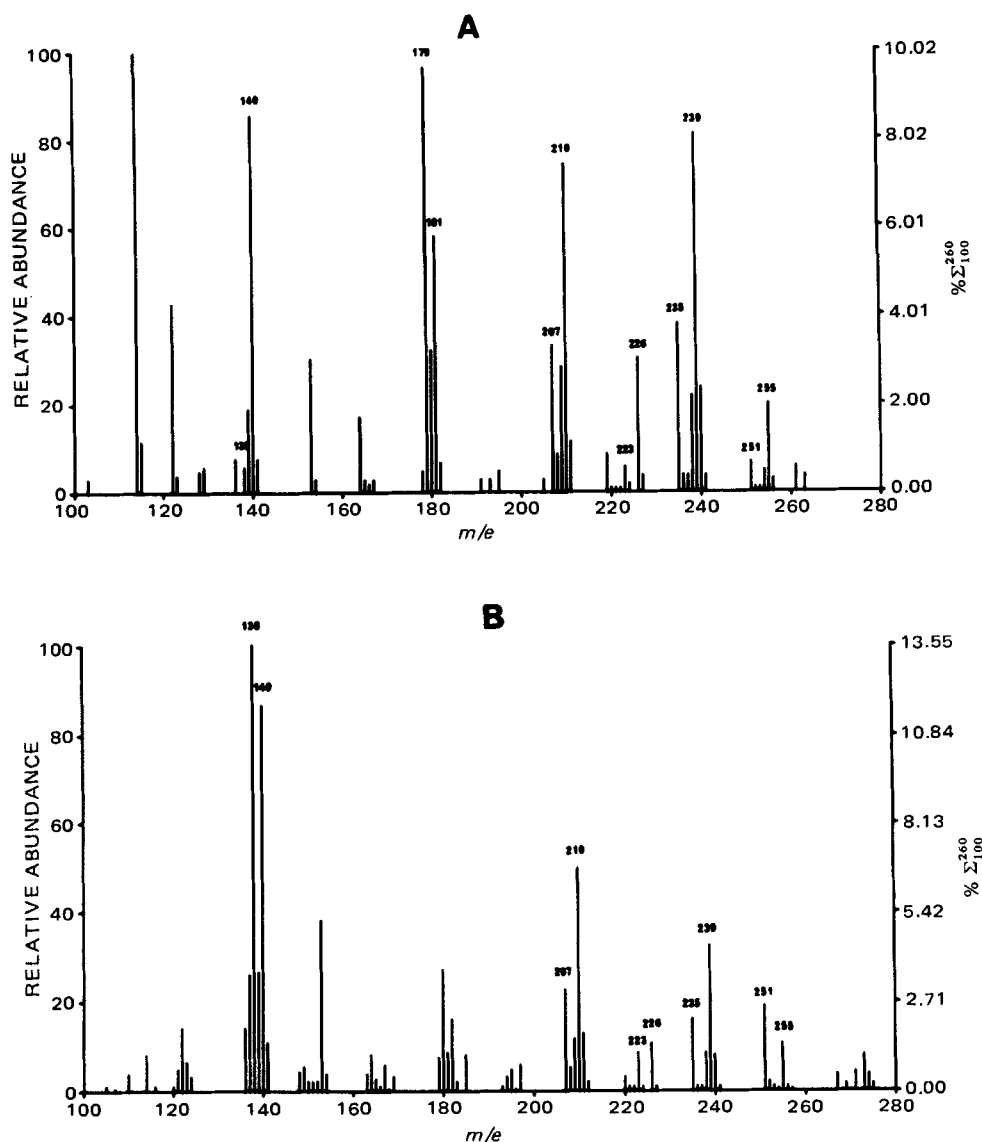


Figure 4—Comparative chemical-ionization mass spectra of the methylene chloride extracts from basified (pH 8.5) 4–8-hr urine samples from Subject C before (A) and after (B) β -glucuronidase-sulfatase treatment. The labeled peaks represent the following metabolites and their deuterated standards: m/e 138, II d; m/e 140, II d-d₂; m/e 179, Ic; m/e 181, Ic-d₂; m/e 207, Ib; m/e 210, Ib-d₃; m/e 223, m- and/or p-Ic; m/e 226, Ic-d₃; m/e 235, Ia; m/e 239, Ia-d₄; m/e 251, m- and/or p-I d; and m/e 255, Id-d₄.

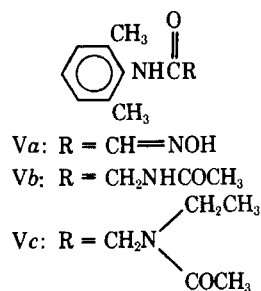
was then stored in a freezer (-15°) until further workup. By using this procedure, any structural change occurring in these metabolites was reflected in identical changes in the added standards. Quantification was carried out before and after β -glucuronidase-sulfatase treatment (Table II). Comparative chemical-ionization mass spectra of the 0–2- and 8–24-hr urine extracts of Subject B are shown in Fig. 3, and comparative spectra of the 4–8-hr urine extracts of Subject C before and after β -glucuronidase-sulfatase treatment are shown in Fig. 4.

Amino Acid Metabolites—The standard isotope dilution methods reported under *Experimental* revealed that minimally *N,N*-diethyl-

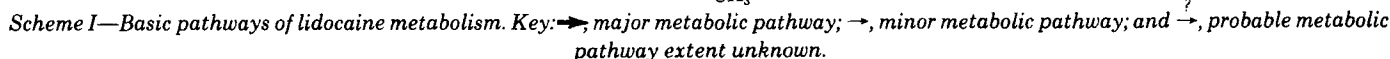
glycine represented 35% of the excreted dose in two human subjects. Although some inexact TLC and chemical-ionization mass spectrometry data indicated that monoethylglycine was also present, this amino acid eluded quantification. However, in a Rhesus monkey, both amino acids were quantified *via* ion-exchange chromatography coupled with GLC and chemical-ionization mass spectrometry analysis²⁰. After receiving 100 mg iv of radiolabeled lidocaine, this monkey excreted a minimum of 15% of IVa and 19% of IVb of the total administered dose.

DISCUSSION

The results of this work show the extreme usefulness of the chemical-ionization mass spectrometry technique in both clinical analysis and drug metabolism studies. By using this procedure in a study of human lidocaine metabolism, several metabolites and their deuterated internal standards could be simultaneously monitored and quantified in a single temperature-programmed analysis. The technique combines speed, accuracy, and specificity in determining and quantifying metabolites; it also offers safety for the investigator. Drawbacks of the method include cost and upkeep of the instrument, time and expense required to synthesize the necessary deuterated standards, and inability to provide in-



²⁰ Unpublished results, this laboratory.



By using the chemical-ionization mass spectrometry-stable isotope dilution technique, 70–80% of lidocaine metabolism in humans can be accounted for. The results support the metabolic sequence outlined by Keenaghan and Boyes (12) with some modifications (Scheme I). As in most species studied, the major primary pathway of lidocaine biotransformation in humans appears to be *N*-dealkylation followed by secondary oxidations, conjugations, and hydrolysis. Any major contributions to lidocaine metabolism by oxidation of the aromatic methyl groups or *N*-hydroxyamide formation (31) were ruled out. However, whereas Keenaghan and Boyes (12) suggested only hydrolysis of the amide linkage after deethylation, the present results indicate that a substantial amount of direct hydrolysis of lidocaine takes place to yield both *N,N*-diethyl- and *N*-monoethylglycine plus 2,6-dimethylaniline, which is further oxidized to *p*-hydroxy-2,6-dimethylaniline, the major urinary metabolites of lidocaine in humans.

- (1) R. P. Lasser, *N. Y. State J. Med.*, **1973**, 1775.
- (2) J. I. Grossman, L. A. Lublow, J. Frieden, and I. L. Rubin, *Arch. Intern. Med.*, **121**, 396 (1968).
- (3) L. Lemberg, A. Castellanos, Jr., A. G. Arecebal, and R. N. V. Iyengar, *Med. Clin. N. Am.*, **55**, 273 (1971).
- (4) J. R. Gillette, in "Microsomes and Drug Oxidation," J. R. Gillette, Ed., Academic, New York, N.Y., 1969.
- (5) K. Krisch, *Biochem. Z.*, **55**, 273 (1963).
- (6) R. G. McMahon and L. A. Woods, *J. Pharmacol. Exp. Ther.*, **103**, 354 (1951).
- (7) C. Y. Sung and A. P. Truant, *ibid.*, **112**, 432 (1954).
- (8) I. C. Geddes, *Anesth. Analg. Reanim.*, **15**, 908 (1958).
- (9) G. Hollunger, *Acta Pharmacol. Toxicol.*, **17**, 356 (1960).
- (10) A. H. Beckett, R. N. Boyes, and P. J. Appleton, *J. Pharm. Pharmacol.*, **7**, 755 (1965).

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Application of Ion-Pair Methods to Extraction of Fluorouracil from Aqueous Fluids

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Abstract □ The polar molecule, fluorouracil, is a monoanion at pH 10 and may be quantitatively extracted from aqueous solutions with quaternary alkylammonium ions into an organic solvent such as dichloromethane as the ion-pair. Extraction constants of fluorouracil with the tetrapentylammonium ion in dichloromethane or dichloromethane-1-butanol (9:1) and with the tetrahexylammonium ion in dichloromethane were determined. Slope analysis demonstrated that association of the ion components in the aqueous phase occurred as the side reaction. A column ion-pair extraction technique, using tetrapentylammonium as the counterion and dichloromethane as the eluting phase, was developed and allowed quantitative transfer of fluorouracil to the organic solvent. The applicability of this method was shown by determining plasma levels of fluorouracil in cancer patients to whom 1 g of active substance was administered intravenously.

Keyphrases □ Fluorouracil—ion-pair extraction from aqueous solutions with quaternary alkylammonium ions into organic solvents □ Ion-pair extraction—fluorouracil from aqueous solutions with quaternary alkylammonium ions into organic solvents □ Antineoplastic agents—fluorouracil, ion-pair extraction from aqueous solutions with quaternary alkylammonium ions into organic solvents

Few methods have been published on the determination of fluorouracil in biological materials. A microbiological assay and a direct spectrophotometric method were used to determine plasma fluorouracil levels in cancer patients (1). Neither of these methods is sufficiently specific. Procedures using GLC were reported (2, 3), and a sensitive and highly specific mass fragmentographic method was recently described (4). However, in all of these techniques, the isolation of fluorouracil from aqueous biological materials into an organic solvent remains a weak point.

Dialysis and subsequent evaporation to dryness (2) and double-phase extraction with 2-propanol-ether (3, 4) were utilized. However, the first method is cumbersome and time consuming, and the two-phase extraction suffers from low specificity. A more polar system is required for extraction of the highly polar fluorouracil, a pyrimidine derivative.

The purpose of the present investigation was to study ion-pair systems, using quaternary alkylammonium counterions, for the extraction of fluorouracil from an aqueous matrix to an organic phase.

EXPERIMENTAL

Apparatus—A pH meter¹ equipped with a combination electrode², a magnetic stirrer³, and a mechanical shaking apparatus⁴ were used. Spectrophotometric measurements were performed with an automatic UV-visible double-beam instrument⁵ using 10-mm quartz cells.

Chemicals and Reagents—Fluorouracil⁶ was used as received. Dichloromethane⁷, tetrapentylammonium iodide⁸, and tetrahexylammonium bromide⁸ were analytical grade. Tetrapentylammonium and tetrahexylammonium hydrogen phosphate solutions, about 0.15 and 0.01 M, respectively, were prepared as follows. To the 0.2 M aqueous solutions of the halides was added an equivalent amount of silver oxide, and the mixtures were continuously stirred for 24 hr. After filtration, concentrated phosphoric acid was added to pH 10. The solutions were extracted three times with small volumes of dichloromethane (phase ratio, aqueous-organic, 10:1) to remove the unreacted iodide and bromide salts (5). The true concentrations of the tetraalkylammonium ions were determined by an ion-pair extraction method with picric acid (6).

Cellulose⁷ for column chromatography was purified in a column by washing with ethanol and dichloromethane until the absorbance of the eluate at 269 nm was constant at 0.010. Carbonate buffer, pH 10 and ionic strength 0.1, was prepared for back-extraction experiments by mixing 25.0 ml of 1 M NaHCO₃ with 50.0 ml of 0.5 M Na₂CO₃ and diluting to 1000 ml. A concentrated carbonate buffer for plasma determinations was made by dissolving 2.1 g of sodium hydrogen carbonate and 2.6 g of sodium carbonate in 40 ml of water.

All other substances were analytical grade and were used without purification. Mutually saturated solvents were used throughout the experiments.

Extraction Constants—Constants for ion-pair extraction and ion-pair association in the aqueous phase were determined by a partition technique. The partition experiments were performed in centrifuge tubes using 20-ml volumes of aqueous and organic solutions. These solutions were mechanically shaken at room temperature for 30 min to establish equilibrium. After centrifugation, the phases were separated and the absorbance of the aqueous phase, A₁, was measured at 269 nm.

A 10.0-ml aliquot of the organic phase was subsequently reequilibrated with 10.0 ml of 0.01 M NaClO₄ in carbonate buffer, pH 10, by shaking for 15 min at room temperature. (A shorter time for establishing the equilibrium was used because of the much higher extraction constant of

¹ Radiometer Titrator TTT 1c, Copenhagen, Denmark.

² Radiometer GK 2321 C, Copenhagen, Denmark.

³ Cenco Instruments N.V. 16632 B, Breda, The Netherlands.

⁴ Wilten and Co., Berchem, Belgium.

⁵ Pye Unicam SP 1800, Cambridge, England.

⁶ Donated by N.V. Produits Roche, Brussels, Belgium.

⁷ E. Merck AG, Darmstadt, West Germany.

⁸ Eastman Kodak, Rochester, N.Y.