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Articles

N-Hydroxyamide Metabolites of Lidocaine. Synthesis, Characterization, Quantitation, and Mutagenic Potential

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Two possible *N*-hydroxyamide metabolites of lidocaine were synthesized and characterized. A combined technique utilizing chemical-ionization mass spectrometry and stable isotope labeling demonstrated that these potentially carcinogenic *N*-hydroxyamides were neither present in human urine after oral lidocaine administration nor during intravenous infusion of lidocaine for the treatment of ventricular arrhythmias. However, small amounts of 2,6-dimethylphenylhydroxylamine were detected in the urine of all subjects. Mutagenesis assays using the Ames test showed that neither the *N*-hydroxyamides nor the *N*-hydroxyarylamine produced revertant colonies above background levels using the *Salmonella* tester strain TA-1538.

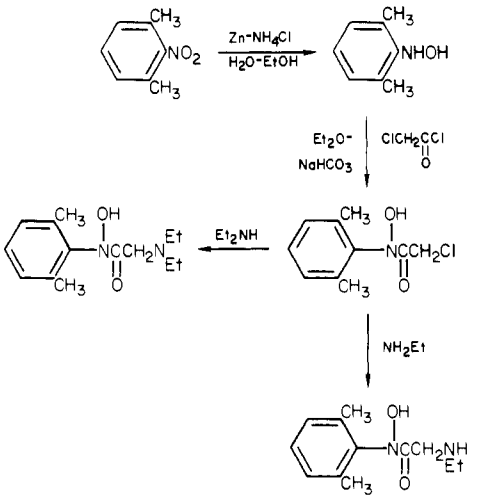
Lidocaine (**1**) is a widely used local anesthetic and antiarrhythmic agent. A major initial metabolite found in man is the mono-*N*-deethylated product MEGX (**2**).[1] Based on indirect evidence, Mather and Thomas[2] have suggested that *N*-hydroxylidocaine (**3**) and *N*-hydroxy-MEGX (**4**) are also major metabolites formed in man after an oral dose of lidocaine (see Chart I). They found that treatment of urine samples with titanium trichloride ($TiCl_3$) caused an increase in the amounts of both lidocaine and MEGX as determined by GC. These results suggested that metabolites containing reducible N–O bonds were present in the urine. They further found that these metabolites were excreted in larger amounts when the urine of the subjects was acidified, suggesting that the oxidation had occurred at the amide nitrogen and not on the more basic amino group.

Because of the carcinogenic propensity of other *N*-hydroxyamides,[3] we undertook the synthesis of **3** and **4** to substantiate the evidence presented by Mather and Thomas and to study further these potentially toxic metabolites. This paper will describe the synthesis and characterization of the *N*-hydroxyamide metabolites and their attempted quantification in urine samples from human subjects who had received lidocaine. Results of tests for mutagenicity will also be described for lidocaine, MEGX, the *N*-hydroxyamides **3** and **4**, and 2,6-dimethylphenylhydroxylamine (**11**).

Results

Synthesis and Characterization. The *N*-hydroxyamides of lidocaine and MEGX were prepared by acylating 2,6-dimethylphenylhydroxylamine with chloroacetyl chloride in a slurry of aqueous sodium bicarbonate in ether and then allowing the ω-chloro-*N*-hydroxyamide to react with the appropriate amine (Scheme I). The *N*-hydroxyamides gave a red color with ferric chloride in ethanol and a violet color with vanadium (V) in chloroform.[5] The reduction of 2,6-dimethylphenylhydroxylamine (**11**), *N*-hydroxylidocaine (**3**), and *N*-hydroxy-MEGX (**4**) with $TiCl_3$ produced 2,6-dimethylaniline (**10**), lidocaine (**1**), and MEGX (**2**), respectively, in quantitative yield. GC analysis of **3**, **4**, and **11** on 3% OV-17 at 185 °C, before or after refluxing in toluene, revealed that these *N*-hydroxy compounds were thermally degraded, primarily to their *N*-deoxy compounds **1**, **2**, and **10**, respectively.

Scheme I. Synthetic Route to *N*-Hydroxyamides



Although the NMR spectra of the *N*-hydroxyamide hydrochlorides of **3** and **4** are consistent with the *N*-hydroxyamide structure, the spectra of the free amines suggest the presence of two isomers, as does the spectrum of their synthetic precursor ω-chloro-2,6-dimethylphenylacetohydroxamic acid (**9**). For example, the NMR spectrum of the hydrochloride salt of *N*-hydroxylidocaine gives the expected triplet and quartet for the *N*-ethyl protons and a singlet at 4.72 ppm for the hydrogens of the methylene group α to the amide carbonyl group. However, the spectrum of the free amine shows two well-resolved

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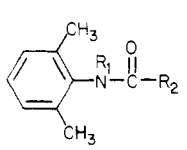
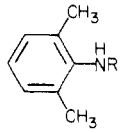
Chart I



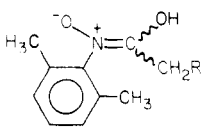


10, R = H
11, R = OH

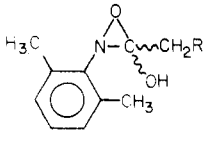
	R_1	R_2
1	H	$CH_2N(CH_2CH_3)_2$
2	H	$CH_2NH(CH_2CH_3)$
3	OH	$CH_2N(CH_2CH_3)_2$
4	OH	$CH_2NH(CH_2CH_3)$
5	H	$CH_2N(CD_2CH_3)_2$
6	H	$CD_2N(CH_2CH_3)_2$
7	OH	$CH_2N(CD_2CH_3)_2$
8	OH	$CH_2NH(CH_2CD_3)$
9	OH	CH_2Cl

sets of triplets for the methyl group hydrogens, two sets of overlapping quartets for the methylene hydrogens of the *N*-ethyl group, and two distinct singlets at 3.55 and 4.60 ppm for hydrogens of the methylene group α to the amide carbonyl.

At the present time, we do not know the structures of the two isomers that are apparently present. Possible geometric isomers of the *N*-hydroxyamides could arise by tautomerization to enamide structures, A, or oxaziridine



A

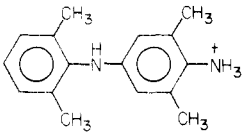


B

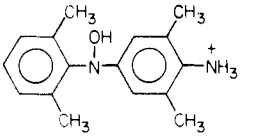
structures, B. The magnetic environments of the structural elements would be different in cis and trans isomers of either tautomer A or B. Enamide structures have been proposed to explain the NMR spectra recorded for *N*-hydroxyureas[6] and oxaziridine structures to explain the GC and MS data for *N*-hydroxy-2-acetylaminofluorene.[7] Work is in progress in our laboratory using ^{13}C NMR to better elucidate the possible nature of the apparent isomers.

Mass spectra of the *N*-hydroxyamides are consistent with the presence of tautomeric structures A or B. Both **3** and **4** showed (M - 16) and (M - 17) ions in the electron-impact (EI) spectra and (MH^+ - 16) and (MH^+ - 18) ions in the chemical-ionization (CI) spectra. The loss of 16 would result from the loss of oxygen under both EI and CI conditions from either the enamide or the oxaziridine ring. The loss of 17 in the EI spectra would result from loss of a hydroxy radical from either structure as well, and the loss of 18 in the CI spectra could represent a loss of water from the same position.

The EI spectrum of 2,6-diphenylhydroxylamine showed a low-intensity molecular ion at *m/e* 137 (2% total ion current = TIC) with an intense ion at *m/e* 121 (M - 16, 18% TIC). The isobutane CI spectrum gave a more intense MH^+ ion at *m/e* 138 (23% TIC) as well as at *m/e* 122 (MH^+ - 16, 56% TIC). Additional ions were present at *m/e* 243 and 259 which probably result from thermal reactions to produce the dimeric ions C and D.



C, *m/e* 243



D, *m/e* 259

Quantitation. The deuterated analogues **5**–**8** were used in a CIMS–stable isotope dilution analysis of human urine samples by the $TiCl_3$ reduction method previously described.[4] The results of the analysis from two subjects who had received an oral dose of lidocaine as the hydrochloride salt and one subject who received an oral dose of lidocaine plus ammonium chloride to maintain a constant urinary pH (5.0–5.3) were virtually identical. The CI mass spectrum of the alkalinilized urine extracts without prior $TiCl_3$ treatment shows only MH^+ ions corresponding to MEGX (*m/e* 207) and MEGX-d_2 internal standard (*m/e* 209) and lidocaine (*m/e* 235) and lidocaine-d_2 internal standard (*m/e* 237). Reaction of the urine with $TiCl_3$ prior to alkalinization reduces the *N*-hydroxy-MEGX-d_3 standard quantitatively to MEGX-d_3 (*m/e* 210) and the *N*-hydroxylidocaine-d_4 standard to lidocaine-d_4 (*m/e* 239). This shows that the $TiCl_3$ treatment worked and at the same time allows for quantitation of *N*-hydroxyamide metabolites by differences in ratios of MEGX (MH^+ *m/e* 207) vs. MEGX-d_2 (MH^+ *m/e* 209) and lidocaine (MH^+ *m/e* 235) vs. lidocaine-d_2 (MH^+ *m/e* 237) before and after $TiCl_3$ treatment. The amounts of MEGX or lidocaine, determined by their MH^+ ions relative to the MH^+ ions of their deuterated standards, were not increased by prior reaction with $TiCl_3$ either before or after β-glucuronidase sulfatase treatment. This same result has been observed with a patient receiving intravenous infusion of lidocaine for the treatment of ventricular arrhythmias.

Another portion of each urine sample was extracted and the *N*-hydroxyamides were separated by two different developing systems. Analysis of the separated material by CIMS showed only the presence of the deuterated *N*-hydroxyamide standards.

Another potential metabolite of lidocaine, 2,6-dimethylphenylhydroxylamine (**11**), was found in all samples of urine that were analyzed. After $TiCl_3$ reduction of urine samples, the amounts of 2,6-dimethylaniline increased, as determined by comparing the ratios of *m/e* 122 (MH^+ of 2,6-dimethylaniline) and *m/e* 124 (MH^+ of [*methyl*-$^2H_2$]-6-methylaniline[8]) before and after $TiCl_3$ treatment. Because of interfering peaks in this region of the spectrum, we could only estimate that about 1% of an administered dose of lidocaine was present in the urine as 2,6-dimethylphenylhydroxylamine. This was confirmed by GC–CIMS analysis using selected ion monitoring of ions at *m/e* 122 and 124, which showed that 1.5% of the dose was present as 2,6-dimethylaniline before and 2.3% of the dose after $TiCl_3$ reduction.

Mutagenicity and Carcinogenicity Testing. Mutagenesis assays using 1, 10, 100, and 500 μg of compounds

1–4, 10, and 11 were performed according to the procedure of Ames and co-workers using tester strain TA-1538.[9] The number of revertant colonies on any plate incubated in the presence of these compounds was never greater than in the absence of compounds (Me_2SO only), which showed 45–60 colonies in the presence of the S-9 fraction or 12–20 colonies in its absence. A positive control, 2-acetylaminofluorene (10 μg), produced 5500 colonies in the presence of the S-9 fraction and background colonies (12–20) without the S-9 fraction. Preliminary carcinogenicity testing of the *N*-hydroxyamides and 2,6-dimethylphenylhydroxylamine through the National Cancer Institute testing program has produced no tumors in Swiss albino mice.

Discussion

The results of our experiments underscore the need for synthetic standards and specific assays for studies in drug metabolism whenever this is possible. GC and MS data obtained with the *N*-hydroxyamides, **3** and **4**, and the *N*-hydroxyarylamine, **11**, show that they decompose primarily by losing oxygen to yield their respective parent compounds **1**, **2**, and **10**. Therefore, if these *N*-hydroxy compounds were metabolites, they would be assayed as the parent compounds using GC procedures, and some of the 2,6-dimethylaniline that has been reported as a metabolite of lidocaine in various species, including man,[1b] may have arisen from the thermolysis of 2,6-dimethylphenylhydroxylamine.

Based on the results of our studies, the *N*-hydroxyamides of lidocaine and MEGX are not major metabolites of lidocaine found in the urine of man. Even if these *N*-hydroxyamides were formed, they would be considered to be nontoxic compared to other *N*-hydroxyamides, such as *N*-hydroxy-2-acetylaminofluorene, which are potent mutagens and carcinogens. Thus, our results support the view that lidocaine is a very beneficial drug with low attendant risk if used as recommended.

Whether or not the *N*-hydroxyamides, **3** and **4**, are metabolites of lidocaine in man, however, is still open to question. Investigations are in progress to determine if measurable levels of *N*-hydroxylidocaine or *N*-hydroxy-MEGX are present in plasma samples obtained from patients receiving iv doses of lidocaine for the suppression of ventricular arrhythmias. The presence of 2,6-dimethylphenylhydroxylamine (**11**) in human urine after doses of lidocaine suggests that this compound either arises by hydrolysis of the *N*-hydroxyamides or by further oxidation of 2,6-dimethylaniline that is formed by enzymatic hydrolysis of either lidocaine or MEGX.[8] This *N*-hydroxyarylamine has been detected in plasma samples of patients on lidocaine therapy,[10] and further studies to clarify the pathway(s) leading to the formation of this metabolite are currently in progress.

Experimental Section

Chemistry. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Infrared spectra were recorded on either a Perkin-Elmer 337 or Varian IR-5A spectrophotometer and NMR on a Varian T-60. Chemical shifts are reported in parts per million relative to Me_4Si. Microanalyses were performed by Huffman Laboratories, Wheatridge, Colo.

EI mass spectra were obtained on an AEI MS 902 (direct inlet, 70 eV); chemical-ionization (CI) mass spectra were obtained on an AEI MS-9 double-focusing mass spectrometer modified for chemical ionization, using isobutane as the reactant gas.[11] All spectra were recorded at an accelerating voltage of 8 kV and a mass resolution of $m/\Delta m$ = 3500 (5% valley definition). GC analyses were obtained on a Varian 2100 gas chromatograph equipped with flame ionization detectors using a 6 ft × 0.25 in. × 2 mm i.d. glass U column packed with 3% OV-17 on 80–100 mesh A/W Chromosorb W. Conditions employed were column temperature variable; injector and detector 250 °C; N_2 carrier gas flow 40 mL/min, hydrogen and air adjusted to give maximum flame response. GC–MS studies were carried out on a VG MicroMass 16F instrument coupled to a Varian Aerograph Model 1400 via a glass-lined tube and a glass jet separator. EI mass spectra were recorded at an accelerating voltage of 4 kV and a mass resolution of $m/\Delta m$ = 1000 (10% valley definition) with an electron energy of 70 eV and a source temperature of 260 °C. GC conditions were the same as previously described except that helium was used as the carrier gas at a flow rate of 25 mL/min, the column temperature was 150 °C, and the interface temperature was maintained at 260 °C. Selected ion monitoring (SIM) was carried out using a programmable multiple ion monitor output to a Rikadenki Model KA-40 four-pen recorder.

The synthesis and purification of compounds 1, **2**, **5**, **6**, and **10** have been previously described.[8]

2,6-Dimethylphenylhydroxylamine (11). To a vigorously stirred solution of 2,6-dimethylnitrobenzene (Aldrich, 15.1 g, 0.10 mol) in 30 mL of absolute ethanol was added, in one portion, a solution of ammonium chloride (7.0 g) in 15 mL of water, followed by small portions of zinc dust (Merck, 94%, 15.0 g). The temperature of the reaction was maintained between 65 and 70 °C by the portionwise addition of zinc dust until all had been added. The warm slurry (55 °C) was suction-filtered and the filter-pak washed with 30 mL of anhydrous ether. Rotary evaporation of the filtrate gave a pale yellow precipitate that was recrystallized twice from purified petroleum ether–benzene (1:1) to yield 12.2 g of white needles: mp 103–104 °C; IR (KBr) 3300 (NH), 3200 cm^{-1} (OH); NMR ($CDCl_3$) δ 2.35 (s, aryl Me's), 6.50 (br s, NH and OH), and 7.05 (s, aromatics). EIMS showed mass fragments at *m/e* 137, 121, 120, 91, 77, 65, 63, 53, 52, and 51 from *m/e* 50 to 300; CIMS showed MH^+ at *m/e* 138 (23% TIC), $MH^+ - 16$ (56% TIC) at *m/e* 122, and dimeric ions at *m/e* 259 (3% TIC) and 243 (18% TIC). Anal. ($C_8H_{11}NO$) C, H, N.

ω-Chloro-2,6-dimethylphenylacetohydroxamic Acid (9). To a magnetically stirred two-phase mixture of 2,6-dimethylphenylhydroxylamine (4.12 g, 0.03 mol) in 100 mL of ether and sodium bicarbonate (2.80 g, 0.034 mol) in 12 mL of water, cooled to −5 °C in an ice–salt bath, was added chloroacetyl chloride (Aldrich, 3.39 g, 0.03 mol) in 20 mL of ether over a 30-min period. The reaction was stirred an additional 20 min at −5 °C and then poured into a separatory funnel and washed with two 50-mL portions of water. 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 of white rhombic crystals: mp 138–140 °C; IR (KBr) 3200 (OH), 1650 cm^{-1} (C=O); NMR ($CDCl_3$) δ 2.10 and 2.32 (s, aryl Me's), 3.76 and 4.41 (s, $OCCH_2Cl$), 7.05 and 7.25 (m, aromatics), and 1.15 (br s, D_2O exchangeable OH). Anal. ($C_{10}H_{12}NO_2Cl$) C, H, N.

ω-(Diethylamino)-2,6-dimethylphenylacetohydroxamic Acid or *N*-Hydroxylidocaine (3). A solution of **9** (2.13 g, 0.01 mol) and diethylamine (Baker, 2.8 mL, 2.2 g, 0.03 mol) in 30 mL of benzene was stirred for 4 h at 65–70 °C. The orange-colored solution was cooled in ice and suction-filtered to remove precipitated diethylamine hydrochloride and then extracted with 3 × 5 mL portions of 10% HCl. The acid extracts were combined and adjusted to pH 8.5 with 5 N NaOH. The opaque mixture was back-extracted with 3 × 20 mL portions of purified chloroform which were combined and washed with 10 mL of water and dried over anhydrous sodium sulfate. Rotary evaporation of solvent at room temperature yielded a pale yellow liquid that was crystallized three times from purified hexane to yield 1.6 g of small white needles: mp 81–82 °C; IR (KBr) 3250 (OH), 1650 cm^{-1} (C=O); NMR ($CDCl_3$) δ 0.85 and 1.15 [t, J = 7 Hz, $N(CH_2CH_3)_2$], 2.30 and 2.35 (s, aryl Me's), 2.50 and 3.00 [overlapped q, J = 7 Hz, $N(CH_2CH_3)_2$], 3.55 and 4.60 (s, $OCCH_2N$), and 7.10–7.20 (m, aromatics plus OH exchangeable with D_2O); high-resolution EIMS calcd for $C_{14}H_{22}N_2O_2$, 250.16812, found 250.16873; mass fragments *m/e* 250, 234, 233, 163, 147, 132, 121, 120, 119, 118, 105, 86; CIMS showed MH^+ at *m/e* 251 (65.1% TIC), *m/e* 235 ($MH^+ - 16$, 19.9% TIC), *m/e* 233 ($MH^+ - 18$, 11.7% TIC), and *m/e* 103 (3.3% TIC) from *m/e* 100 to 300. Anal. ($C_{14}H_{22}N_2O_2$) C, H, N.

The hydrochloride was made by adding the calculated amount of concentrated HCl to a solution of 3 in 2-propanol and cooling

the solution in a freezer to give small white crystals: mp 182–184 °C; IR (KBr) 2450 (OH), 2700 (NH), 1665 cm^{-1} (C=O); NMR (acetone-d_6) δ 1.45 [t, J = 7 Hz, $N(CH_2CH_3)_3$], 2.30 (s, aryl Me's), 3.55 [q, J = 7 Hz, $N(CH_2CH_3)_2$], 4.09 (br s, NH and OH), 4.72 (s, $OCCH_2N$), and 7.23 (s, aromatics).

ω-(Diethylamino-α,α-d_4)-2,6-dimethylphenylacetohydroxamic Acid or *N*-Hydroxylidocaine-d_4 (7). This deuterated analogue was synthesized by the same procedure as 3 using diethylamine-d_4: mp (HCl) 182–184 °C.

ω-(Ethylamino)-2,6-dimethylphenylacetohydroxamic Acid or *N*-Hydroxy-MEGX (4). A solution of **9** (2.13 g, 0.01 mol) and anhydrous ethylamine (Eastman, 1.95 mL, 1.35 g, 0.03 mol) in 30 mL of purified *p*-dioxane was stirred for 4.5 h at 50–55 °C. 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. The filtrate was evaporated and the residue recrystallized twice from benzene to yield 1.4 g: mp 103–105 °C; IR (KBr) 3250 (OH), 1650 cm^{-1} (C=O); NMR ($CDCl_3$) δ 1.05 and 1.17 (t, J = 7 Hz, NCH_2CH_3), 3.00 and 3.85 (s, $OCCH_2N$), 5.95 (s, OH and NH exchangeable with D_2O), and 7.10 and 7.20 (s, aromatics); high-resolution EIMS calcd for $C_{12}H_{18}N_2O_2$, 222.13726, found, 222.13682; mass fragments m/e 222, 206, 205, 163, 147, 132, 121, 120, 119, 118, 105, 91, 77, 58, 56; CIMS showed MH^+ at m/e 223 (66.8% TIC), m/e 207 (MH^+ – 16, 19.2% TIC), m/e 205 (MH^+ – 18, 12.0%), and m/e 75 (2.0% TIC) from m/e 70 to 300. Anal. ($C_{12}H_{18}N_2O_2$) C, H, N.

The hydrochloride was made by adding the calculated amount of concentrated HCl to a solution of 4 in 2-propanol and cooling the solution in a freezer to give small white crystals: mp 187–188 °C; IR (KBr) 3450 (OH), 2700–2900 (NH), and 1665 cm^{-1} (C=O); NMR (acetone-d_6) δ 1.40 (t, J = 7 Hz, NCH_2CH_3) 2.35 (s, aryl Me's), 3.35 (q, J = 7 Hz, NCH_2CH_3), 4.25 (br s, NH and OH), 4.50 (s, OCH_2N), and 7.20 (s, aromatics).

ω-(Ethylamino-β-d_3)-2,6-dimethylphenylacetohydroxamic Acid or *N*-Hydroxy-MEGX-d_3 (8). This deuterated analogue was synthesized by the same procedure as the undeuterated compound using ethylamine-d_3: mp (HCl) 185–187 °C.

Chemical and Chromatographic Properties. Solutions were prepared of 2.5 mg of the hydroxyamides, 3 and 4, and the *N*-hydroxyarylamine, 11, in 10 mL of water, and 2 mL of a second solution [concentrated HCl (1 mL), 20% $TiCl_3$ (6 mL), and water (5 mL)] was added. Each solution was heated for 10 min at 50 °C and then basified to pH 12 with 5 N NaOH and extracted with 30 mL of diethyl ether. After drying the ether extract over $CaSO_4$, the solvent was evaporated under reduced pressure and the residue was dissolved in 1 mL of methanol. A 50-μL portion of the reconstituted solution was chromatographed on silica gel GF plates (Eastman No. 6060) using cyclohexane–chloroform–methanol (70:30:10) as the developing solvent. Only the reduced compounds 1 (R_f 0.78), **2** (R_f 0.45), and **10** (R_f 0.90) were detected by short-wave UV after $TiCl_3$ reduction of **3** (R_f 0.37), 4 (R_f 0.25), and 11 (R_f 0.68), respectively. A 2-μL portion of each reconstituted solution was analyzed by GC on 3% OV-17 (column temperature, 185 °C). As determined from standard curves of varying amounts of compounds 1, **2**, and **10** (retention times 9.5, 8, and 1 min, respectively) using the procedure described above, **3**, **4**, and 11 had been quantitatively reduced to their corresponding *N*-deoxygenated compounds.

Either direct GC analysis or refluxing solutions of **3**, 4, and 11 in toluene prior to GC analysis produced degradation products. The major components (>75%) of the degradation products were the deoxygenated compounds 1, **2**, and **10**, respectively.

CIMS–Stable Isotope Dilution Analysis. The procedure for analyzing the *N*-hydroxy compounds of lidocaine in human urine samples is detailed elsewhere.[4] In addition to the studies already reported, urine and heparinized blood samples were obtained at 30 h from a consenting patient in a coronary care unit (Bethesda Naval Hospital, Bethesda, Md.) who received intravenous lidocaine (1.4 mg/min) for 36 h for suppression of ventricular arrhythmias. The plasma levels of lidocaine (1) and MEGX (2) were monitored by a CIMS assay previously described.[1d] Lidocaine levels were found to be 3.1 ± 0.4 μg/mL and MEGX levels were 0.68 ± 0.07 μg/mL at this time.

Aliquots of the patient's urine were analyzed immediately after collection as described for the oral administration studies already reported.[4] Another aliquot ($^1/_{10}$) of the urine, that contained 0.5 mg of the deuterated standards **7** and **8**, was carefully adjusted to pH 8.5 with 5 N NaOH and extracted with 3 vol of methylene chloride. The methylene chloride was back-extracted with 0.5 N ammonium hydroxide. This extract was carefully acidified to pH 8.0 with 2 N HCl and reextracted into 3 vol of methylene chloride. Rotary evaporation of the solvent yielded a residue that was taken up in 1 mL of methanol and 100 μL was applied to a 5 × 20 cm × 500 μ silica gel GF plate (Analtech) and developed in cyclohexane–chloroform–methanol (70:30:10). A second plate was developed in cyclohexane–benzene–methanol (5:90:10). Those areas corresponding to the R_f of the *N*-hydroxyamides were visualized with short-wave UV due to the presence of the deuterated internal standards. The areas were scraped, eluted with methanol, and subjected to CIMS analysis using a direct insertion probe. Only ions that were due to the presence of the deuterated standards, **7** (MH^+ m/e 255) and **8** (MH^+ m/e 226), were observed. This same result was found if the urine was hydrolyzed with Glusulase (Endo Laboratories) prior to the extraction procedure.

Urine samples that contained known amounts of methyl-2-d_2-6-methylaniline were analyzed for 2,6-dimethylaniline by adjusting aliquots ($^1/_{10}$ of total urine) to pH 12.0 with 5 N NaOH and extracting with 3 vol of diethyl ether, followed by back extraction into 2 N HCl. The acid extracts were then adjusted to pH 10.0 with 5 N NaOH and reextracted into 2 vol of ether. Rotary evaporation of the ether yielded a residue that was taken up in 1 mL of methanol and 2 μL was analyzed by GC–CIMS and monitoring the ions at m/e 122 and 124 and the MH^+ ions of 2,6-dimethylaniline and its dideuterated analogue. This procedure was carried out before and after $TiCl_3$ treatment of the urine samples.

Mutagenesis Experiments. Mutagenesis assays were performed according to Ames and co-workers.[9] To 2 mL of molten top agar at 45–47 °C was added 0.15 mL of the *Salmonella* tester strain TA-1538 (2×10^9–3×10^9 bacteria/mL), 0.1–0.5 mL of Me_2SO containing the chemical to be tested, and 0.5 mL of the S-9 mixture containing (per milliliter) 0.3 mL of S-9 fraction, 8 μmol of $MgCl_2$, 33 μmol of KCl, 5 μmol of glucose 6-phosphate, 4 μmol of NADP, and 100 μmol of sodium phosphate buffer, pH 7.4. The colonies on each plate (histidine revertants) were counted after a 2-day incubation at 37 °C. The liver fraction (S-9) was prepared by the method of Felton et al.[12] and consisted of a 1:4 homogenate prepared in sterile 100 μM sodium phosphate buffered 0.85% NaCl (pH 7.2) from the livers of C57BL/6J mice that had received a single dose of 3-methylcholanthrene (80 mg/kg, ip) in corn oil 40 h prior to sacrifice.

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Metabolic Fate of Tolazamide in Man and in the Rat

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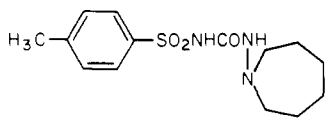
The metabolic fate of tolazamide, 1-(hexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (1), was studied in man and in the rat using tritium-labeled 1. The metabolites were isolated in crystalline form from urine for structure determination. The crystal structure and final molecular structure of one of these, 1-(4-hydroxyhexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (**5**), were determined using single-crystal X-ray techniques. Following oral administration of tritiated tolazamide to male humans, 85% of the radioactivity was excreted in urine during a 5-day period. In addition to being excreted in urine unchanged, tolazamide was metabolized to 1-(hexahydroazepin-1-yl)-3-*p*-(carboxyphenyl)sulfonylurea (**2**), *p*-toluenesulfonamide (3), 1-(hexahydroazepin-1-yl)-3-*p*-(hydroxymethylphenyl)sulfonylurea (**4**), 1-(4-hydroxyhexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (**5**), and a labile, unidentified metabolite **6** by man. The relative amounts of these materials excreted in 0–24-h urine collections from eight subjects averaged 7, 17, 26, 10, 25, and 15% for 1–**6**, respectively. In the female rat, 79% of an orally administered dose of tritiated tolazamide was excreted in urine during a 5-day period as 1–4. The relative amounts of these materials excreted during the 24-h period following administration of tolazamide were 10, 5, 5, and 80% for 1–4, respectively.

The biotransformation of tolazamide,[1] 1-(hexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (**1**), an orally active,



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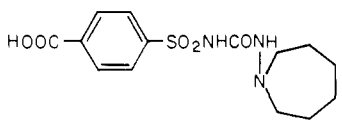
hypoglycemic agent, has not been studied extensively. Forist and Judy[2] reported the isolation and identification of 1-(hexahydroazepin-1-yl)-3-*p*-(carboxyphenyl)sulfonylurea (**2**) as a urinary metabolite of tolazamide in the



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human; this is the only prior report concerning biotransformation of the drug.

The present work, employing tritium-labeled tolazamide, was undertaken to determine both qualitatively and quantitatively the metabolism of this drug in the human and rat.

Experimental Section

Materials. The preparation of tritium-labeled tolazamide by exposure of nonradioactive 1 to tritium gas has been described.[3] Its chemical and radiochemical purities were ascertained immediately prior to its use. Although the intramolecular distribution of tritium in tritium-labeled 1 was not determined, a similar compound (tolbutamide), also prepared by tritium gas exposure, contained 85% of its tritium in the aromatic ring.

Authentic, synthetic samples of tolazamide (1), *p*-toluenesulfonamide (3), and *p*-toluenesulfonylurea, as well as an authentic sample of 1-(hexahydroazepin-1-yl)-3-*p*-(carboxyphenyl)-sulfonylurea (**2**) which had been isolated from human urine by Forist and Judy,[2] were used as chromatographic and spectral standards.

Radioactivity Measurements. All counting was performed with a Packard Tricarb Model 314EX2A liquid scintillation spectrometer at −8 °C under conditions suitable for measuring tritium. Appropriate aliquots of samples were dissolved in 15 mL of scintillation solvent [toluene–dioxane–MeOH (350:350:210 by volume) containing 73 g of naphthalene, 4.6 g of 2,5-diphenyloxazole, and 0.08 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter]. The absolute counting efficiency for each sample was determined by recounting following addition of an internal standard of tritium-labeled toluene, and results were then converted to disintegrations per minute.

Radiochromatography. Paper chromatography was carried out in the *n*-BuOH–piperidine–H_2O (81:2:17 by volume) system on Whatman No. 1 paper. Dried chromatograms were routinely examined under short-wavelength UV light through a fluorescent screen to locate standards and, when possible, metabolites by fluorescence quenching. Zones of radioactivity were located and quantified by cutting the developed paper strip into sequential 1.25-cm segments and counting the segments in individual vials using scintillation solvent containing 3% H_2O.

Thin-layer chromatography was carried out in the $CHCl_3$–MeOH–formic acid (95:4:1 by volume), $CHCl_3$–EtOAc–formic acid (50:48:2 by volume), and $CHCl_3$–EtOH–heptane–formic acid (33:33:33:0.5 by volume) systems on 0.25-mm films of silica gel GF. The UV absorption of standards and, when possible, metabolites was detected by viewing the dried chromatograms under short-wavelength UV light. The zones of radioactivity were located and quantified by transferring sequential 0.5-cm segments of the developed chromatogram into individual vials and counting using scintillation solvent containing 3% H_2O.

Instrumental Analysis. Electron-impact and field-desorption mass spectra were obtained with a Varian MAT CH5 instrument by direct probe techniques. Ultraviolet analyses were conducted with a Cary 15 spectrometer using 95% EtOH as a solvent. A Perkin-Elmer 421 infrared spectrometer was used to analyze samples in the form of Nujol mulls. Nuclear magnetic resonance spectra were obtained with Varian A-60A and XL100 instruments using acetone-d_6 and DMF-d_7 as solvents. Melting points were determined with a Thomas-Hoover capillary melting point apparatus. Optical rotation was measured with a Bellingham and Stanley polarimeter. Single-crystal X-ray diffraction data were obtained with a Syntex P$\bar{1}$ diffractometer controlled by an IBM 1800 computer.

Single-Dose Administration of Tolazamide to Rats and Collection of Samples. Each of three female, Sprague–Dawley rats, weighing approximately 215 g, was given a 3.8-mg (8.6 μCi) dose of tritium-labeled tolazamide, as a solution of its ammonium salt, by stomach tube. The rats were housed in individual metabolism cages, designed for the separation and collection of urine and feces, and allowed food and water ad libitum during the 5-day study. Each daily urine collection was analyzed for total radioactivity and tritiated water. Each daily fecal collection was analyzed for total radioactivity by an oxygen-flask combustion method.

Preliminary Extraction and Characterization of Rat Metabolites. The 0–24-h urine collections from this study were combined, saturated with $(NH_4)_2SO_4$, acidified with H_2SO_4, and

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