ISOLATION AND IDENTIFICATION OF CLOZAPINE METABOLITES IN PATIENT URINE

GISELA SCHABER, GERLINDE WIATR, HELMUT WACHSMUTH, MARKUS DACHTLER, KLAUS ALBERT, INES GAERTNER, AND URSULA BREYER-PFAFF

Department of Toxicology (G.S., U.B.-P.), Psychiatric Clinic (G.W., I.G.), and Department of Organic Chemistry (M.D., K.A.), University of Tuebingen, Tuebingen, Germany; and Department of Analytics, Boehringer Ingelheim Pharma, Biberach, Germany (H.W.)

(Received November 28, 2000; accepted February 13, 2001)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:

Biotransformation products of the atypical neuroleptic clozapine were isolated from urine samples of three schizophrenic patients by solid-phase extraction, liquid–liquid extraction for the separation of unpolar and polar metabolites, and thin-layer chromatography followed by final purification by high-performance liquid chromatography. Their structures were elucidated by mass spectrometry and 1H NMR spectroscopy and in some cases by enzymatic deconjugation. Besides the known metabolites desmethylclozapine, clozapine N-oxide, 8-deschloro-8-hydroxyclozapine, and 8-deschloro-8-hydroxydesmethylclozapine, the unpolar fraction contained 7-hydroxyclozapine and a compound in which the piperazine ring of clozapine was partially degraded to an ethylenediamine derivative. Novel metabolites identified in the polar fraction were the sulfate and glucuronide conjugates of 7-hydroxyclozapine N-oxide, 8-deschloro-8-hydroxyclozapine-O-glucuronide, and the O-glucuronide of N-hydroxydesmethylclozapine; further conjugates were tentatively identified as 9-hydroxyclozy methylclozapine-O-sulfate and 6-hydroxyclozapine-O-sulfate. In addition, the previously described conjugates 7-hydroxyclozy methylclozapine-O-sulfate, 7-hydroxyclozapine-O-glucuronide and -O-sulfate, 8-deschloro-8-hydroxydesmethylclozapine-O-glucuronide, and the quaternary ammonium glucuronide of clozapine were detected.

Clozapine [2-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e]1,4-diazepine] [1,4]-diazepine] was the first neuroleptic drug to reveal clinical properties that later were used to define the group of atypical neuroleptics (Coward, 1992). On the other hand, clozapine therapy bears a risk of agranulocytosis of 1 to 2%, and this adverse effect is assumed to be mediated by a chemically reactive metabolite (Pirmohamed et al., 1995). Therefore, a complete knowledge of biotransformation routes undergone by clozapine is desirable. The metabolism of clozapine (CLZ)3 in vitro by human liver microsomes and/or expressed human cytochrome P450 enzymes has been the subject of several investigations (Fischer et al., 1992; Pirmohamed et al., 1995; Eiermann et al., 1997; Limnet and Olesen, 1997; Tugnait et al., 1997; Fang et al., 1998), but except for unknown metabolites produced by CYP2D6, the investigations were confined to the so-called “major” biotransformation products desmethylclozapine (DMCLZ) and clozapine N-oxide (CLZ-NO), which had originally been identified in patient urine by Gauch and Michaelis (1971). Measurements in patient urine revealed, however, that these two together accounted for only 14% of the dose (Scherab e et al., 1998), such that additional metabolites were expected to contribute markedly to the total fate. An analysis of patient urine for unconjugated basic compounds resulted in the identification of DMCLZ, CLZ-NO, and of minor products in which the chloro substituent was replaced by a hydroxy or methylthio group (Stock et al., 1977).

An attempt to elucidate the whole pattern of clozapine metabolites in human urine and feces has been limited to volunteers given a single dose of [14C]clozapine (Dain et al., 1997). It led to the detection in urine of 7-hydroxyclozy methylclozapine-O-sulfate and the glucuronides of 7-hydroxyclozapine (previously identified in rat bile by Zhang et al., 1996) and 8-deschloro-8-hydroxydesmethylclozapine. In addition, the quaternary ammonium glucuronide of clozapine, which had previously been isolated from patient urine (Luo et al., 1994), could be measured in feces (Dain et al., 1997). Since the authors used a single HPLC step for metabolite separation, the possibility had to be considered that minor metabolites were overlooked.

The present investigation aimed at elucidating as completely as possible the pattern of unconjugated and conjugated clozapine metabolites in the urine of patients under continuous monotherapy by using sequential separation steps.

Experimental Procedures

Materials. Clozapine, N-desmethyloclozapine, and clozapine N-oxide as reference substances were kindly provided by Novartis Pharma (Basel, Switzerland). Clozapine N’-glucuronide was prepared according to Luo et al. (1992), but the derivatization reaction was carried out at 42°C, and the product was isolated by passing the extracted aqueous phases through cartridges with C18 Silica gel (Bond Elut, Varian, Harbor City, CA) and eluting with methanol. The eluted N’-glucuronide was purified by thin-layer chromatography as described below for polar metabolites. β-Glucuronidase/arylsulfatase from Helix pomatia and β-glucuronidase from Escherichia coli K12 were purchased from Roche Diagnostics (Mannheim, Germany).

Patients. Urine samples were collected by three in-patients of the Psychiatric Clinic, University of Tuebingen, suffering from schizophrenia. Patient 1...
was a 35-year-old male weighing 80 kg. He was treated with 350 mg/day clozapine for his psychosis and with prednisolone for idiopathic thrombocytopenic purpura. On the day of urine collection, five serum clozapine measurements performed at 2-h intervals resulted in 411 ± 87 ng/ml, while desmethyloclozapine amounted to 275 ± 62 ng/ml. His 24-h urine sample of 1700 ml was completely processed. Patient 2, a 19-year-old male weighing 59 kg, was treated with 600 mg/day clozapine and had clozapine and desmethyloclozapine serum levels of 1950 ± 110 and 995 ± 45 ng/ml, respectively. Metabolite analyses were done on a 2-h urine fraction of 395 ml. Patient 3, a 31-year-old male, had serum concentrations of 331 ng/ml clozapine and 283 ng/ml desmethyloclozapine under treatment with 400 mg/day clozapine. Of his 24-h urine (3050 ml), two 400-ml portions were processed.

**Isolation of Metabolites.** Urine samples of 300 to 600 ml were applied at a rate of 5 ml/min to a C18 silica gel column (150 × 10 mm, Polygosil 40–63 µm, Macherey-Nagel, Düren, Germany) pretreated with methanol and water. After washing with 100 to 150 ml of water, substances were eluted with 100 to 150 ml of 0.1 M acetic acid in methanol, and the solvent was evaporated under reduced pressure. The residue was separated into unpolar bases and polar substances by addition of 50 ml of water, pH adjustment to 9 with 25% aqueous ammonia and four extractions with 50 ml of tert-butyl methyl ether. The organic phases were evaporated, and unconjugated metabolites contained in the residue were dissolved in 0.5 ml of methanol and separated by thin-layer chromatography on four to six sheets of 20 × 20 cm precoated with 0.25 mm of silica gel with fluorescent indicator (Alugram SIL G/UV 254, Macherey-Nagel). Solvent I, composed of 2-propanol/tert-butyl methyl ether/25% ammonia/water (5:5:1.5:0.5, v/v), resulted in 10 to 151 ml from which the metabolites were extracted three times with 2 ml of methanol. These fractions were subjected to a separation in solvent III, 1-butanol/acetic acid/water (4:1:1, v/v), followed by the same extraction procedure.

The resulting extracts were purified by HPLC.

**Purification by HPLC.** The system consisted of a 200- × 4.6-mm column filled with C18 silica gel 5 µm (Nucleosil 5 C18, Macherey-Nagel) coupled to a photometric detector (UVIS 205, Linear) with which spectra could be recorded during the run. The usual detection wavelength was 290 nm. The solvents run at 1 ml/min were mixtures of 0.02 M ammonium acetate in 0.9 M acetic acid (pH 3.0) and methanol in the ratios of 50:50, 70:30, 80:20, or 90:10 (v/v) according to the polarity of the metabolites. Spectra were recorded for all peaks and eluates were collected. Eluates with spectra resembling that of clozapine were evaporated under reduced pressure. The purified compounds were dissolved in methanol and ESI-MS, MS/MS was measured along with the average m/z for clozapine (0.21 at 10 µg/ml). Estimates of metabolite quantities were obtained on the assumption of equal molar absorptivity.

**Enzymatic Hydrolysis.** Quantities of conjugated metabolites of about 20 µg were dissolved in 0.4 ml of 0.1 M sodium acetate buffer, pH 5, and incubated with 100 µl of β-glucuronidase/arylsulfatase from H. pomatia for 24 h at 37°C. Incubates were alkalized with ammonia and liberated compounds extracted three times with 2 ml of tert-butyl methyl ether, and in some cases subsequently with ethyl acetate. Extract residues were subjected to TLC in solvent I, if possible in parallel with known unconjugated metabolites. Clozapine liberated from its N′-glucuronide by the same procedure or by an excess of β-glucuronidase from E. coli in phosphate buffer, pH 7, within 2 h was detected in the above HPLC system with 50% methanol in the eluent, resulting in a retention time of 8.5 min.

**Mass Spectrometry.** Mass spectra in the electron-impact mode (EI-MS) were recorded with a double-focusing mass spectrometer (Finnigan MAT 8230 with Al/DEVA) with a combined EI/CI ion source (ThermoFinnigan, San Jose, CA). Substances were directly introduced into the ion source, and spectra were recorded over the range 20 to 750 amu at a rate of 3 s/decade, an ionization energy of 70 eV, and an acceleration voltage of 3000 V.

A TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT) and Finnigan acquisition software were used for spectra in the electrospray ionization (ESI) and collision-induced dissociation (CID) modes. The samples were dissolved in methanol/water (9:1) to concentrations of 10 to 20 ng/µl. These solutions were infused via a syringe pump at a flow rate of 1.5 µl/min into the ion source. The positive and negative ion electrospray needle voltages were +4500 and −3500 V, respectively. The temperature of the heated transfer capillary was set to 120°C. Sheath gas was nitrogen. Spectra were acquired at 1.5 to 2 s/decade over 1 min, and the recorded spectra were averaged.

In the CID-MS mode, argon was used as collision gas. The collision cell pressure was 1.9 to 2 mtorr, and the collision offset voltages were between −20 and −35 eV for positive ions and between 22 and 35 V for negative ions. Spectra were recorded at 1.5 to 2 s/decade and processed by the ICIS release 8.1 software (Finnigan MAT).

**1H NMR Spectrometry.** Spectra were recorded in a 400-MHz instrument (AMX 400, Bruker) with a 5-mm dual probe at 300 to 320 K. Substances were dissolved in deuterated methanol with 99.8% deuterium. The solvent signal at 4.86 ppm served for standardization. For processing of recorded spectra, the interferogram of the free induction decay with 16-k data points was multiplied with a line broadening of 0.3 Hz.

**Results**

**Unpolar Fraction.** On TLC of organic-extractable compounds from patient urine, seven bands were obtained that according to their UV spectra contained clozapine metabolites. Each of these resulted in up to four peaks in HPLC. Structures of metabolites that were isolated and identified are shown in Fig. 1.

CLZ, DMCLZ, and CLZ-NO were identified by their Rp values in TLC, their retention times in HPLC (Table 1), and their 1H NMR spectra (Table 3) and mass spectra in comparison with those of authentic substances. DMCLZ exceeded all other unpolar compounds in quantity, whereas the major part of CLZ-NO was recovered in the polar metabolite fraction. In mass spectrometry, CLZ-NO either lost oxygen to form CLZ with identical subsequent degradation (Stock et al., 1977), or it underwent a Cope elimination losing 60 amu (C14H16NO) and 74 amu (C12H18NO).

The most abundant phenolic metabolite was 8-deschloro-8-hydroxydesmethylclozapine (8-OH-DMCLZ), as reported by Dain et al. (1997). Its mass spectrum was in accordance with that published by Stock et al. (1977). The 1H NMR spectrum confirmed the 8-position of the hydroxy group because the coupling pattern of aromatic protons was unchanged relative to that in clozapine (Table 3). The signals of H-7 (6.38 ppm) and H-9 (6.48 ppm) neighboring the OH group were strongly shifted up-field compared with those in clozapine.

The N-methylated analog 8-deschloro-8-hydroxyclozapine (8-OH-CLZ) was present in smaller quantities in patient urine (Table 1). Its mass spectra were analogous to those of 8-OH-DMCLZ with [M + H]+ 309 in ESI-MS and losses characteristic of a methylated piperazine ring, i.e., 70, 83, and 99 amu from the molecular ion m/z 308 in EI-MS. The signal of the N-CH3 group was present at 2.36 ppm in the 1H NMR spectrum, and the coupling patterns and chemical shifts of the aromatic protons closely resembled those in 8-OH-DMCLZ (Table 3).

A small quantity of unconjugated 7-hydroxyclozapine (7-OH-CLZ) was detected in urine of patient 1. In ESI-MS, the presence of CI resulted from m/z 343/345 with the typical isotope pattern for the [M + H]+ ion; the EI-MS could not be interpreted because of contamination. In 1H NMR, the protons of the unsubstituted aromatic ring gave the same signals as in clozapine, whereas those of the substituted ring appeared as two singlets at 5.61 and 6.81 ppm, indicative of a marked up-field shift (Table 3). Thus, an electron-donating substituent...
must have been introduced into position 7 in addition to the Cl in position 8.

Partial metabolic degradation of the piperazine ring led to a novel metabolite, 2-chloro-11-(2-methylamino-ethylamino)-5H-benzodiazepine, an ethylenediamine derivative (EDA-BZD) present in urine of patient 1. Its structure was deduced from a chlorine-containing [M + H]+ 301/303 in ESI-MS and fragment ions in EI-MS resulting from losses of 44 amu (C2H6N), 57 amu (C3H7N), and 73 amu (C3H9N2) from the molecular ion m/z 300/302. The quantity was not sufficient for 1H NMR spectrometry.

**Polar Fraction.** As mentioned above, a major compound in the fraction not extractable into tert-butyl methyl ether was CLZ-NO

---

*Fig. 1. Structures of clozapine metabolites in patient urine and possible metabolic pathways for their production.*

\[ R = \text{H for free phenols; } R = \text{glucuronosyl } (C_6H_9O_6) \text{ for glucuronides; } R = \text{SO}_3\text{H for sulfates.} \]
All other metabolites isolated from this fraction proved to be conjugates with glucuronic acid or sulfenic acid (Fig. 1).

In all three patients, 7-hydroxydesmethyclozapine-O-sulfate (7-OH-DMCLOZ-O-Sulf) previously described by Dain et al. (1997) by far exceeded other conjugates in quantity (Table 2). The loss of 80 amu (SO$_3$) from the molecular ion 408/410 was followed by a loss of 69 amu (C$_{6}$H$_{7}$Cl) from the unmethylated piperazine ring to m/z 259/261. No N-CH$_3$ signal was present in the $^1$H NMR spectrum. As in 7-OH-CLZ, resonances of H-1 to H-4 were nearly the same as in clozapine, while H-6 and H-9 appeared as singlets, in this case H-6 with a strong down-field shift to 7.15 ppm caused by the neighboring sulfite ester (Table 3). The signals of H-2, H-4, and H-9 between 6.99 and 7.04 ppm were superimposed on one another, but integration clearly indicated the presence of three protons.

The homologous 7-hydroxylozapine-O-sulfate (7-OH-CLZ-O-Sulf) was also detected in all urine samples, but in much smaller quantities than the demethylated compound (Table 2). Its CID-MS/MS was analogous with [M + H]$^+$ 423, from which m/z 343 originated by loss of SO$_3$ and m/z 286 by subsequent piperazine ring fragmentation (343 - C$_2$H$_7$N). The EI-MS contained m/z 342/344 (as M + SO$_3$) and m/z 272/274 and 259/261 resulting from piperazine ring fragmentation. The $^1$H NMR spectrum (Table 3) was nearly identical with that of 7-OH-DMCLOZ-O-Sulf, but a N-CH$_3$ signal was present at 2.43 ppm.

The corresponding glucuronide, 7-hydroxylozapine-O-glucuronide (7-OH-CLZ-O-Gluc), was detected only in urine from patient 3. In the negative ion ESI-MS mode, the presence of Cl was documented by [M – Cl]$^-$ 517/519 in accordance with the sum formula C$_{24}$H$_{27}$ClN$_4$O$_7$. In CID-MS/MS, the [M – H]$^-$ ion m/z 517 eliminated 176 amu (glucuronic acid – H$_2$O) to give m/z 341. Additional fragments resulted from admixture of a glucuronide of hydroxy-DMCLOZ. The position of the O-glucuronide resulted from two singlets at 6.93 and 6.98 ppm in the $^1$H NMR spectrum corresponding to H-6 and H-9, respectively. The N-CH$_3$ group gave a signal at 2.37 ppm (Table 3).

Conjugates were also formed from 7-hydroxylated CLZ-NO. Thus, 7-hydroxylozapine-N-oxide-O-sulfate (7-OH-CLZ-NO-O-Sulf) was present in urine from patients 1 and 3. In CID-MS/MS, [M + H]$^+$ appeared at m/z 439/441 and [M – H]$^-$ at 437/439 (Fig. 2); the latter lost CH$_3$ resulting in m/z 422/424 and further SO$_3$ to give m/z 342/344, which is consistent with the presence of a sulfite ester. Direct removal of SO$_3$ from [M – H]$^-$ led to m/z 357/359. In EI-MS, fragments were formed that corresponded to those of CLZ + 16 amu as a result of aromatic ring hydroxylation. In $^1$H NMR (Fig. 3), the N-CH$_3$ signal was found at 3.25 ppm in accordance with its chemical shift in CLZ-NO. The resonances of the aromatic protons were nearly identical with those in 7-OH-DMCLOZ-O-Sulf (Table 3).

The analogous glucuronide, 7-hydroxylozapine-N-oxide-O-glucuronide (7-OH-CLZ-NO-O-Gluc), was excreted by patient 3. In accordance with the sum formula C$_{24}$H$_{27}$ClN$_4$O$_8$, the ESI-MS exhibited [M + H]$^+$ 535/537, [M + Na]$^+$ 557/559, and in the negative ion mode [M – H]$^-$ 533/535. In ESI-MS/MS positive ion mode, the piperazine ring was fragmented as in CLZ-NO, with losses from m/z 535 of 18 amu (H$_2$O) and 100 amu (C$_6$H$_5$NO); alternatively, 176 amu (glucuronic acid – H$_2$O) and 194 amu (18 + 176) were lost to give the corresponding positive fragment ions. The base peak at m/z 315 resulted from successive losses of 176 and 44 amu (C$_5$H$_7$NO) and a prominent peak at m/z 259 from losses of 176 and 100 amu. The $^1$H NMR spectrum confirmed the structure by showing the N-CH$_3$ signal at 3.23 ppm. The pattern of aromatic proton signals was identical with that in 7-OH-CLZ-O-Gluc, while the chemical shifts showed minor differences (Table 3).

Conjugated 8-hydroxy metabolites were recovered as glucuronides only. A small quantity of 8-deschloro-8-hydroxylozapine-O-glucuronide (8-OH-CLZ-O-Gluc) was found in urine from patient 3. The absence of Cl resulted from mass spectrometry with [M + H]$^+$ 485, [M + Na]$^+$ 507, and [M – H]$^-$ 483 in ESI-MS. CID-MS caused the loss of 176 amu (glucuronic acid – H$_2$O) from [M + H]$^+$ to form m/z 309, which lost 57 amu (C$_6$H$_5$N) with production of m/z 252. In the negative ion mode, [M – H – 176]$^-$ appeared as m/z 307. In $^1$H NMR, the presence of a glucuronosyl group could not be demonstrated directly. However, the somewhat higher chemical shifts of H-6, H-7, and H-9 in comparison with those in unconjugated 8-OH-CLZ (Table 3) are clearly in favor of a glucuronide. The identity of the metabolite was further proven by enzymatic hydrolysis to an aglycon that in TLC in solvent I had the same $R_F$ (0.55) as 8-OH-CLZ from the unpolar fraction (Table 1).

A moderate quantity of the demethylated analog 8-deschloro-8-hydroxydesmethyclozapine-O-glucuronide (8-OH-DMCLOZ-O-Gluc) was excreted by patient 3. Its mass spectral data differed from those of 8-OH-CLZ-O-Gluc by 14 amu and confirmed the loss of 176 amu from the molecular ion (Dain et al., 1997). The $^1$H NMR spectrum was in accordance with the structure, inasmuch as here, too, the H-6, H-7, and H-9 signals were shifted down-field in comparison with 8-OH-DMCLOZ (Table 3). Further confirmation came from enzymatic degradation to 8-OH-DMCLOZ with $R_F$ 0.26 in TLC-solvent I in accordance with that of the metabolite in the unpolar fraction (Table 1).

For two further phenolic sulfates, structure assignment remained tentative. One of these excreted by patient 1 was identified as 9-hydroxydesmethyclozapine-O-sulfate (9-OH-DMCLOZ-O-Sulf). In CID-MS/MS, it did not differ from the 7-hydroxy analog, but in $^1$H NMR
spectrometry a different pattern of aromatic protons in the substituted ring became apparent. The signals of H-6 at 7.13 ppm and of H-7 at 6.97 ppm appeared as doublets with J = 4.69 Hz. Since coupling is only possible between H-6 and H-7, the hydroxy group must have been introduced at C-9. Uncertainty existed because the J value was below the calculated value of 8 Hz for vicinal protons, although it was higher than would be expected in the case of meta coupling. Proton signals between 6.97 and 7.03 ppm were superimposed, but integration indicated contributions from three protons (H-2, H-4, and H-7). No N-CH₃ signal was discernible due to superimpositions.

A further metabolite found in urine of patient 3 was assigned the structure of 6-hydroxyclozapine-O-sulfate (6-OH-CLZ-O-Sulf). The composition resulted from [M + Na]⁺ 445/447 and [M − H]⁻ 421/423 in ESI-MS. In MS/MS mode, m/z 421 lost 80 amu (SO₃). ¹H NMR resonances of a basic N-CH₃ group were not detected. The pattern of aromatic proton signals closely resembled that of 7-OH-CLZ-O-Sulf with a slight difference in chemical shifts (Table 3). The possibility that the two metabolites were identical could be excluded, based on differences in Rₚ values in TLC in solvent III and in Rₜ in HPLC. Calculation of aromatic proton resonances for 6-OH-CLZ-O-Sulf gave good agreement with the experimental data.

All three patients excreted small quantities of clozapine N⁺-glucuronide (CLZ-N⁺-Gluc). It was identical with the synthetic N⁺-glucuronide with respect to Rₚ values in TLC, Rₜ in HPLC, mass and NMR spectra, and liberation of CLZ on treatment with β-glucuronidase from H. pomatia or E. coli. The UV spectra of synthetic CLZ-N⁺-Gluc and clozapine were identical, which confirmed the site of glucuronic acid attachment. In CID-MS, [M + H]⁺ 503 either lost 176 amu (glucuronic acid − H₂O) to form the base peak at m/z 327 or 100 amu (C₆H₁₂N₂, corresponding to the methylated piperazine ring) resulting in m/z 403. The latter fragmentation must have involved an intramolecular rearrangement with attachment of the glucuronic acid residue to the C=N bond of the central ring. The base ion m/z 327 exhibited the fragmentation pattern of clozapine with loss of 31 and 57 amu. ¹H NMR spectrometry revealed minor influences on the chemical shifts of the aromatic protons. In Hartman-Hahn rotating-frame nuclear Overhauser effect spectroscopy, the anomic proton H-1⁺ exhibited coupling with other protons of the glucuronide residue and with protons of the piperazine ring. Due to superimposition of the water signal, the N⁺-CH₃ protons were not discernible, but signals of a basic N-CH₃ group were clearly missing.

A novel metabolite was N-OH-DMCLZ-O-Gluc isolated from urine of patients 1 and 3. In CID-MS (Fig. 4), [M + H]⁺ 505
\( \text{(C}_2\text{H}_{25}\text{ClN}_4\text{O}_7) \) was fragmented by loss of 176 amu (glucuronic acid \(-\text{H}_2\text{O}) \) or 194 amu (glucuronic acid) to \( m/z \) 329 or 311, respectively. Therefore, the glucuronosyl residue must be attached through oxygen. The base peak \( m/z \) 243 was the same as that of DMCLZ and resulted from removal of 86 amu (C\(_4\)H\(_8\)NO) from \( m/z \) 329, indicating that hydroxylation and glucuronidation have taken place at the piperazine ring. The \( ^1\text{H} \) NMR spectrum (Fig. 5) showed a pattern of aromatic protons identical with that of DMCLZ, with small up-field shifts of the signals of H-4, H-6, and H-9. No N-CH\(_3\) signal was discernible, and the piperazine ring protons appeared at 2.88 ppm (H-3a and H-5a) and 3.4 ppm (H-2a and H-6a). No signal splitting occurred, which would have indicated carbon atom substitution.

In some metabolite samples, mass and NMR spectra exhibited signals produced by a contaminant commonly used as a solvent stabilizer. This compound, bis-(2-methyl-5-tert-butyl-4-hydroxyphenyl)-sulfide, has a molecular ion \( m/z \) 358 and a fragment ion \( m/z \) 343 in EI mass spectrometry. In \( ^1\text{H} \) NMR, its presence could be recognized by peaks at 6.59 and 6.88 ppm; signals between 3.1 and 3.7 ppm were superimposed on those of the piperazine ring and glucuronosyl protons in some clozapine metabolite spectra and made an exact assignment of peaks impossible.

**Discussion**

A large variety of clozapine metabolites was detected in patient urine, most of the minor ones not having been described previously. Introduction of OH with removal of Cl at C-8 was confirmed as a major pathway leading to 8-OH-CLZ (Table 1; Stock et al., 1977), 8-OH-DMCLZ, and its O-glucuronide (Tables 1 and 2; Stock et al., 1977; Dain et al., 1997). The reaction can be regarded as loss of halogen from the \( para \) position of an aromatic amine and may proceed via a quinoneimine as a reactive intermediate (Rietjens et al., 1990). This opens the possibility of glutathione conjugation, but the respective conjugates were detected neither by Dain et al. (1997) nor in the present work.

Another quantitatively important biotransformation was hydroxylation at C-7 giving rise to a glucuronide in rat bile (Zhang et al., 1996) and human urine (Table 2; Dain et al., 1997). The sulfuric acid conjugate of 7-OH-DMCLZ was described as a major metabolite in human urine (Dain et al., 1997) after it was tentatively identified in rat bile (Zhang et al., 1996). Additional 7-hydroxylation products were unconjugated 7-OH-CLZ (Table 1), its sulfate conjugate, which had been identified in rat bile by its mass spectrum (Zhang et al., 1996), and the sulfuric and glucuronic acid conjugates of 7-OH-CLZ-NO. The only phenolic N-oxide derivatives of another tricyclic psychoactive drug hitherto described are the glucuronide and sulfate conjugates of 7-hydroxyfluperlapine N-oxide that were detected in the rat in vivo and in hepatocyte cultures (Paine et al., 1984; Dain and Jaffe, 1988) and in cultures of human hepatocytes (Guillouzo et al., 1988); however, a description of rigorous structural identification is missing. Clozapine and fluperlapine, a close structural analog of clozapine, are distinguished by a prominent role of N-oxidation in their biotransformation.

Another novel finding was a metabolite tentatively identified as 9-OH-DMCLZ-O-Sulf (Table 2). A phenolic sulfate showing \( ^1\text{H} \) resonances in accordance with those calculated for 6-OH-CLZ-O-Sulf indicated that hydroxylation of CLZ occurred also at the 6-position. In addition to aromatic hydroxylation, DMCLZ was hydroxylated at N-4 of the piperazine ring, and the resulting hydroxylamine was conjugated with glucuronic acid. The structure of the conjugate could be deduced by mass spectrometry from the molecular ion and from the loss of glucuronic acid including an oxygen through which it was attached. Fragmentation also led to loss of the piperazine ring in addition to the glucuronosyl group, but not to piperazine ring removal alone. \( ^1\text{H} \) NMR confirmed that the anomeric proton of the glucuronosyl residue appeared at 4.65 ppm, indicative of a pronounced up-field shift relative to the signal in phenolic glucuronides. Anomeric protons with similar shifts were detected in the glucuronides of hydroxylamine metabolites of a benzazepine (4.84 ppm, Straub et al., 1988) and of mexiletine (4.54 ppm, Turgeon et al., 1992).
Fig. 3. $^1$H NMR spectrum (400 MHz) of 7-hydroxyclozapine-N-oxide-O-sulfate recorded in methanol-d$_4$ (intense signals at 4.86 and 3.25 ppm).

The upper trace of numbers shows the parts per million values of the $^1$H NMR signals, and the lower trace shows the corresponding frequency values in Hertz. The signal at 6.9 ppm (*) is due to an impurity.

Fig. 4. CID-MS/MS of the cation of N-hydroxysmethyloclozapine-O-glucuronide.
As in the investigation of Luo et al. (1994), the occurrence of a quaternary ammonium glucuronide of clozapine could be demonstrated, and attachment of the glucuronosyl group to the terminal nitrogen of the N-methylpiperazine ring was additionally confirmed by Hartman-Hahn rotating-frame nuclear Overhauser effect spectroscopy. In analogy to the N-g-glucuronides of other tertiary amine drugs (Fischer and Breyer-Pfaff, 1995; Mey et al., 1999; Kowalczyk et al., 2000), the clozapine metabolite was hydrolyzed by β-glucuronidase from H. pomatia as well as from E. coli. In contrast, the tertiary N-glucuronide derived from the closely related olanzapine was resistant to these enzymes (Kassahun et al., 1997).

Partial degradation of the piperazine ring to the ethylenediamine derivative EDA-BZD was not unexpected in view of analogous biotransformation reactions in piperazine-substituted phenothiazines (Breyer et al., 1974) and antihistamines (Gaertner et al., 1973). The ethylenediamine derivatives accumulated in animal organs on chronic drug administration (Gaertner et al., 1973, 1975) and their urinary excretion by patients was sustained for several weeks after termination of treatment (Breyer and Gaertner, 1973). Experiments in rats have not revealed accumulation of the respective CLZ metabolites (G. Wiatr, H. Gaertner, and U. Breyer-Pfaff, unpublished results). Metabolites resulting from substitution of the methylthio or methylsulfone group for Cl at C-8 (Stock et al., 1977) were not found in the present analyses nor in those reported by Dain et al. (1997).

The present investigation did not aim at quantitation of the metabolites. These were purified by several sequential steps for which recoveries have not been measured. The rough estimates of quantities given in Tables 1 and 2 are meant to mirror relative amounts. Absolute values were considerably lower than those published by Dain et al. (1997) based on a single HPLC separation. On the other hand, peaks obtained by these authors may have contained minor metabolites in addition to the major ones that were identified.

The present results indicate that in addition to the major hydroxylation products at C-7 and C-8 of the aromatic system, patients treated with clozapine excrete minor ones substituted at the terminal nitrogen of the piperazine ring and at C-6 and C-9 of the ring system. The latter phenols may be analogs of the C-6 and C-9 glutathione derivatives that originated via a nitrenium ion on incubation of clozapine with hypochlorous acid or activated human neutrophils (Liu and Uetrecht, 1995). For phenols produced in parallel, the exact position of the substituents could not be determined. The authors stressed the possibility that oxidation to a nitrenium ion is the first step to covalent binding, which may result in clozapine-induced agranulocytosis. Thus, the tentative identification of 6- and 9-hydroxylated metabolites in urine is the first indication of such a bioactivation pathway in patients, possibly occurring in neutrophils.

In conclusion, isolation of clozapine metabolites from patient urine by sequential chromatographic steps and structural elucidation by instrumental analysis led to the detection of several hitherto unknown products resulting from hydroxylation of CLZ, DMCLZ, or CLZ-NO at C-7 or C-8 and probably also at C-6 or C-9 followed by conjugation with glucuronic or sulfuric acid, from glucuronidation of a hydroxylamine derivative of DMCLZ and from partial degradation of the...
piperazine ring. It is not known whether any of these metabolites is of pharmacological or toxicological importance.

Acknowledgments. We thank M. Cavegn, E. Endris, and U. Fischer (Boehringer Ingelheim Pharma, Biberach) for measuring NMR and mass spectra and Dr. A. Ding and Dr. K. Wagner for the opportunity to use the analytical instruments. We are grateful to Dr. H. Händel, Department of Organic Chemistry, University of Tübingen, for expert interpretation of the NMR spectra.

References


Acknowledgments. We thank M. Cavegn, E. Endris, and U. Fischer (Boehringer Ingelheim Pharma, Biberach) for measuring NMR and mass spectra and Dr. A. Ding and Dr. K. Wagner for the opportunity to use the analytical instruments. We are grateful to Dr. H. Händel, Department of Organic Chemistry, University of Tübingen, for expert interpretation of the NMR spectra.