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Characterization of metabolites of clozapine N-oxide in the rat by micro-column high performance liquid chromatography/mass spectrometry with electrospray interface

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Abstract

The metabolism of clozapine N-oxide was investigated in the rat ($n = 6$) after a single oral dose of 20 mg kg^{-1}. The organic extracts of rat urine were separated by conventional high performance liquid chromatography (HPLC) and individual collected fractions were analyzed by micro-column electrospray HPLC/mass spectroscopy. The compounds identified in rat urine were clozapine N-oxide, clozapine, *N*-desmethylclozapine, 8-deschloro-8-hydroxyclozapine, 8-deschloro-8-thiomethylclozapine, *N*-desmethylclozapine, 8-deschloro-8-hydroxyclozapine, 8-deschloro-8-thiomethylclozapine, *N*-desmethyl-8-deschloro-8-thiomethylclozapine and 8-deschloro-8-methylsulfinylclozapine. With the exception of the unchanged clozapine N-oxide, no other metabolite containing a N-oxide functional group could be found. the concentrations of clozapine N-oxide, clozapine and *N*-desmethylclozapine excreted from rat urine were determined utilizing a conventional HPLC procedure with UV detection. The recoveries of these three analytes reported as the percentage of the dosage from the 0.24 h urine are $0.93 \pm 0.54\%$, $0.06 \pm 0.03\%$ and $0.01 \pm 0.006\%$ respectively.

Keywords: Clozapine; Clozapine N-oxide; Electrospray MS; HPLC/MS; Metabolism; N-oxide reduction

1. Introduction

It is well established that N-oxidation is one of the major routes of metabolism for drugs with a tertiary amine functional group [1, 2], and that the resultant N-oxide metabolites can undergo significant reduction back to their parent drugs in a number of species including rat [3–5], dog [6] and human [7–9]. It has been reported that the N-oxide metabolite of chlorpromazine was shown in healthy volunteers, rats and dogs to be converted back to chlorpromazine [10–12]. The metabolic interconversion between N-oxide

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metabolites and their parent drugs in vivo is important in terms of the therapeutic activity of drugs with an aliphatic tertiary amine functional group. In the case of the tricyclic antidepressant drugs imipramine and amitriptyline, extensive investigations of their N-oxide metabolites have resulted in the marketing of these N-oxide compounds as antidepressants in some countries [13,14] whereas chlorpromazine N-oxide was active only after conversion back into the parent drug in vivo [15].

Clozapine is an atypical antipsychotic drug. Clozapine N-oxide (**I**, Fig. 1) is one of the major metabolites of clozapine (**II**. Fig. 1) [16,17]. In early studies of the metabolites of clozapine in mice, dogs and humans [16], the approximate concentration ratio of clozapine, *N*-desmethylclozapine (**III**, Fig. 1) and clozapine N-oxide determined in patient urine was 1:1:2, although only 7% of the administered clozapine dose was recovered in the urine as clozapine N-oxide in recent studies [18]. The plasma concentration of clozapine N-oxide has been detected in psychiatric patients and ranged from 10–25% [19] to 10–50% [20] of the total clozapine concentration. Although several reports have described the kinetics of clozapine [16,19,17,21–23], with the exception of some work on *N*-desmethylclozapine [24,25], little attention has been paid to the role of metabolites including clozapine N-oxide. There may be metabolic reduction of clozapine N-oxide to clozapine, as has been demonstrated in vivo with chlorpromazine N-oxide in humans [11] and animals [10,12]. Clozapine N-oxide may be important in terms of its contribution in therapeutic activity of clozapine. There is only one published abstract which reported the determination of blood levels of clozapine and its N-oxide after oral and intravenous administrations of either clozapine or clozapine N-oxide to a dog [26]. In this study the conversion of clozapine N-oxide back to clozapine was shown after administration of clozapine N-oxide via both routes, and the maximum concentration of clozapine N-oxide after administration of clozapine was reached more rapidly than the maximum level of clozapine after administration of clozapine N-oxide. However, the ratio of concentrations of clozapine and clozapine N-oxide oscillated during the first 6 h after administration and pharmacokinetic model analysis was not accomplished. Since there has been no systematic investigation of the metabolism of clozapine N-oxide, the present study was carried out to characterize the metabolites in urine after administration of a single oral dose of clozapine N-oxide to rats and also to determine the quantities of clozapine N-oxide and its major metabolites, including clozapine, excreted in urine.

2. Materials and methods

2.1. Chemicals

Solvents used for extraction and for the preparation of the high performance liquid chromatography (HPLC) mobile phase were HPLC grade obtained from BDH Chemicals Canada Ltd. (Edmonton, Alb., Canada). Chemical reagents were commercial analytical grade purchased from Aldrich Chemical Company (Milwaukee, WI). Clozapine and authentic standards of clozapine N-oxide and *N*-desmethylclozapine were kindly supplied by Sandoz Inc. (East Hanover, NJ) and Sandoz Pharmaceuticals (Dorval, Que., Canada).

2.2. Chemical synthesis

2.3.1 Clozapine N-oxide

To a solution of clozapine (10.181 g, 31.23 mmol) in tetrahydrofuran (10 ml) at −70°C was added 3-chloroperoxybenzoic acid (8.057 g, 46.85 mmol). After stirring the reaction mixture for 30 min it was treated with diethylamine (5.07 ml, 48.80 mmol) for 10 min to destroy the excess 3-chloroperoxybenzoic acid. The reaction mixture was slowly brought to room temperature after which the solvent was removed on a rotary evaporator. The crude product was purified by flash column chromatography over silica gel (230–400 mesh) using a mixture of methanol, dichloromethane and ammonium hydroxide (25:200:2, v:v) as eluent. The appropriate fraction was collected and solvents were removed on a rotary evaporator. The residue was crystallized from ethyl acetate. Clozapine N-oxide was ob-

tained (9.159 g, 86%) as yellowish crystals; m.p. 248–249°C (authentic sample m.p. 246–248°C); TLC R_f 0.06 (identical to the authentic standard, EtOH–CH_2Cl_2–NH_4OH, 3:6:0.1); HPLC analysis yields a single peak with a retention time of 14.5 min (identical to the authentic standard, CH_3CN–MeOH–0.1 M NH_4OH, 82:10:8, 1.5 ml min^{-1}); IR (KBr) ν_{max} (cm^{-1}) 1610: (aromatic ring), 1568 (NO), 1470 (CH_3 asym.), 1380 (CH_3 sym.); ^{1}H-NMR (CD_3OD, 300 MHz) δ: 7.38 (1H, dt, 1.34, 7.66 Hz, H-2), 7.32 (1H, dd, 1.36, 7.85 Hz, H-4), 7.09 (1H, dt, 0.92, 7.66 Hz, H-3), 7.00 (1H, dd, 0.92, 7.66 Hz, H-1), 6.97 (1H, d, 2.33 Hz, H-9), 6.87 (1H, dd, 2.32, 8.42 Hz, H-7), 6.80 (1H, d, 8.63 Hz, H-6), 3.84–3.57 (6H, m, piperazinyl 2H-3, 2H-5, H-2e, H-6e), 3.32 (3H, s, CH_3), 3.14 (2H, m, piperazinyl H-2a, H-6a); ^{13}C-NMR

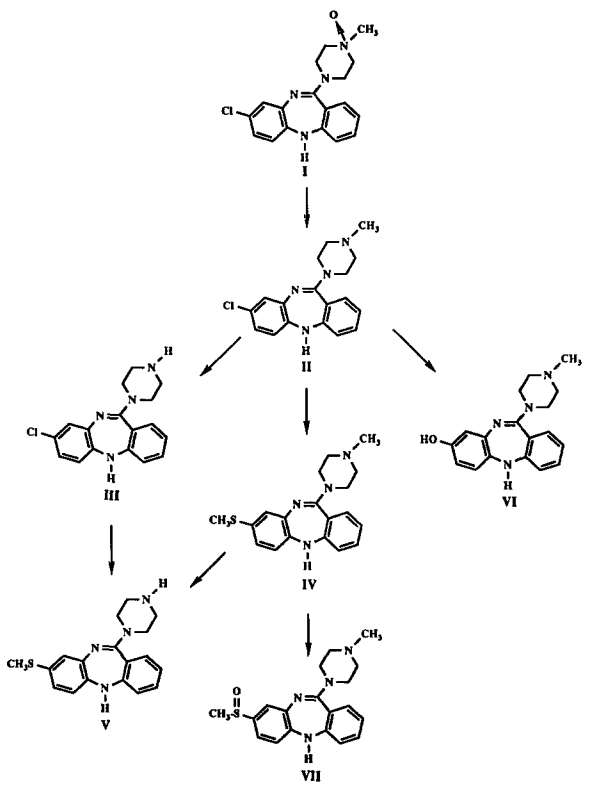


Fig. 1. Metabolic pathway of clozapine N-oxide in rat: **I**, clozapine N-oxide; **II**, clozapine; **III**, *N*-desmethylclozapine; **IV**, 8-deschloro-8-thiomethylclozapine; **V**, *N*-desmethyl-8-deschloro-8-thiomethylclozapine; **VI**, 8-deschloro-8-hydroxyclozapine; **VII**, 8-deschloro-8-methylsulfinylclozapine.

(CD_3OD, 125.8 MHz) δ: 163.9 (s, C-11), 155.5 (s, C-4a), 143.2 (s, C-5a), 143.8 (s, C-9a), 133.7 (d, C-3), 131.1 (d, C-1), 129.6 (s, C-11a), 127.3 (d, C-7), 124.9 (d, C-2), 124.2 (d, C-9), 123.9 (s, C-8), 121.5 (d, C-6), 121.4 (d, C-4), 658 (2C, t, piperazinyl C-3, C-5), 60.2 (q, CH_3), 49.0 (2C, t, piperazinyl C-2, C-6, overlapped with the solvent signals); MS (FAB^+) *m/z* (relative intensity %); 343/345 (MH^+, 100/38), 327/329 ($MH^+ - 16$, 32/11), 325/327 (26/32), 296/298 (13/6), 282/284 (12/7), 270/272 (21/7), 256/258 (43/16), 244/246 (21/9), 243/245 (63/29), 229 (29), 228 (20), 227 (33), 193 (22), 192 (64), 117 (15), 99 (27), 98 (10), 97 (18), 94 (11); MS (ESI^+, sampling cone voltage 90 V) *m/z* (relative intensity %); 365/367 (MNa^+, 25/8), 343/345 (MH^+, 100/36), 325/327 (10/3), 312/314 (3/1), 256/258 (20/7), 243/245 (40/28), 191 (79), 99 (89). The elemental composition of the nominal mass measured at 343 yields the formula $C_{18}H_{20}ClN_4O$ which represents the protonated molecular ion of mono-oxygenated clozapine with a deviation of 0.6 mmu from the theoretical accurate mass. The accurate mass measured was 343.1318 while the theoretical accurate mass is 343.1324.

2.4. *N*-Desmethylclozapine

Clozapine (3.2 g, 9.8 mmol) was dissolved in chloroform (60 ml). To this solution was added 2,2,2-trichloroethylchloroformate (4 ml, 28 mmol) and *N*,*N*-diisopropylethylamine (5 ml, 28 mmol). The mixture was heated under reflux for 3 h. The solvents were removed under reduced pressure and the residue was taken up into 90% acetic acid (20 ml). To the acidic solution was added zinc dust (1.2 g) and the mixture was then stirred at room temperature for 3 h. The zinc was filtered off and the solution was basified by sodium hydroxide and then extracted with dichloromethane. The crude extracts were purified by flash column chromatography over silica gel utilising dichloromethane and methanol (10:1, v:v) as eluent. The collected appropriate fractions were evaporated under reduced pressure. The residue was recrystallized from a mixture of methanol and water, then recrystallized from ethyl acetate, *N*-desmethylclozapine was obtained (1.25 g, 41%) as

yellowish crystals; m.p. 168–169°C (authentic sample m.p. 168–169°C); TLC R_f 0.36 (identical to the authentic standard, EtOH–CH_2Cl_2–NH_4OH, 3:6;0.1): HPLC analysis yielded a single peak with a retention time of 7.5 min (identical to the authentic standard, CH_3CN–MeOH–0.1 M NH_4OH, 82:10:8, 1.5 ml min^{-1}; IR (KBr) ν_{max} (cm^{-1}): 1600, 1500 (aromatic ring): ^{1}H-NMR (CD_3OD, 300 MHz) δ: 7.29–6.62 (7H, m, aromatic), 4.90 (1H, s, ^{5}NH), 3.49 (5H, m, piperazinyl 2H-2, 2H-6, ^{4}NH), 3.02 (4H, piperazinyl 2H-4, 2H-5); MS (EI^+) m/z (relative intensity %): 312/314 (M^+, 37/15), 282/284 (11/4), 268/270 (17/9), 256/258 (57/21), 243/245 (100/36), 227/229 (32/15), 192 (55), 85 (8): MS (ESI^+, sampling cone voltage 45 V) m/z (relative intensity %): 313/315 (MH^+, 100/33); elemental analysis for $C_{17}H_{17}ClN_4$: Calc., C: 65.20; H: 5.43; N: 17.90: Found, C: 65.08; H: 5.54; N: 17.96.

2.5. Instrumentation

2.5.1. Conventional HPLC analysis

The HPLC system consisted of a solvent delivery pump (Waters, model 510), a valve loop injector loop injector with 200 ml loop (Model 7125, Rheodyne), a variable-wavelength UV spectrophotometer (Waters, model 480) set at 257 nm with a sensitivity range of 0.01 absorbance unit full scale (AUFS), and equipped with a Spherisorb (SPE Limited, Rexdale, Ont.) cyano column (3 μm, 150 mm × 4.6 mm i.d., packed in-house using a Shandon column packer, SPE Limited). The data were recorded using a Shimadzu integrator (Model C-R3A, Shimadzu Corporation, Kyoto, Japan). The mobile phase was composed of 90% acetonitrile and 10% 0.08 M aqueous ammonium acetate solution and was degassed by filtration before use (HVLP-type membrane filters, Millipore Canada Ltd., Mississauga, Ont., Canada). The high performance liquid chromatograph was operated at ambient temperature and at a mobile phase flow rate of 1.2 ml min^{-1}.

2.5.2. Electrospray mass spectrometry (MS) and micro-column HPLC/MS

Electrospray ionization (ESI) mass spectra were carried out on a VG Bio-Q triple quadrupole mass spectrometer (VG Biotech, Altrincham, UK). The HPLC solvent delivery system utilized a model 140A dual syringe pump (Applied Biosystems, Mississauga, Ont., Canada) fitted with a Rheodyne 7125 injection valve loop injector equipped with a 10 μl sample loop. The microcolumn (100 mm × 0.5 mm i.d.) was packed with 3 mm Spherisorb cyano packing (packed in-house). The mobile phase was composed of 90% methanol, 10% doubly-distilled water and 1% 0.1 M ammonium accetate with a flow rate of 6 ml min^{-1}. Data were recorded utilizing an Intel 386-based data system using LAB-BASE. Spectra obtained in a direct loop injection were collected at a sampling cone voltage of 45 V in a positive continuum mode at approximately unit mass resolution with consecutive scans summed, typically 15–20 scans. Full scan mass spectra from HPLC/MS were obtained, at sampling cone voltages of either 45 V or 80 V, in the peak centroid mode over the mass range of m/z 50–700 at a rate of 4 s per scan. The individual samples were dissolved in an appropriate volume of acetonitrile and an aliquot of 1 μl was injected using the valve loop injector.

2.6. Metabolic studies

2.6.1. Protocols

Female ($n = 3$) and male ($n = 3$) Lewis rats (body weight 210–370 g) were dosed by gastric intubation with 20 mg kg^{-1} of clozapine N-oxide in aqueous solution. The animals were placed in individual metabolic cages immediately after drug administration. Food was withheld for 24 h after administration, but access to water was allowed at all time. Urine was collected for 24 h after drug administration.

2.7. Characterization of metabolites in urinary organic extracts

Aliquots of urine samples from each species were individually lyophilized (Labconco Freeze Dryer-18, Fisher Scientific and Co., Edmonton, Alb. Canada) and extracted with methanol (1 × 5 ml, 2 × 2 ml). The combined methanolic extracts were dried. The residue was dissolved in 2 ml of

water and 0.5 ml of 0.5 M sodium carbonate was added. The samples were extracted with 15% pentane and 5% isopropanol in ethyl acetate 2 × 5 ml) and then with ethyl acetate (5 ml) for 15 min. The combined organic extracts were dried. The residue was reconstituted in acetonitrile and analyzed by conventional HPLC. The fractions corresponding to the appropriate peaks of the HPLC chromatograms were collected and dried under nitrogen. The residues were reconstituted in acetonitrile and aliquots were directly analyzed by ESI mass spectrometry and/or on-line micro-column HPLC/MS.

In the analysis of hydroxylated metabolites, the appropriate fraction collected from HPLC was dried, dissolved in acetonitrile (50 ml) and incubated with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-*N*-trifluoroacetamide (MTBSTFA, 15 ml) at 65°C for 10 min. The resultant mixture was dried under nitrogen, dissolved in acetonitrile and analyzed by ESI micro-column HPLC/MS.

2.8. Quantitation of clozapine N-oxide, clozapine and N-desmethylclozapine

Standard solutions in blank rat urine (2 ml) at concentrations of 1, 5, 10, 25 and 50 mg ml^{-1} of clozapine N-oxide, 0.1, 0.2, 0.5, 1.0 and 1.5 mg ml^{-1} of clozapine and 5, 10, 50, 100 and 500 ng ml^{-1} of N-desmethylclozapine were prepared in triplicate, to which was added 10 ml acetonitrile solution containing 1 mg of the internal standard (promazine). To each 2 ml aliquot of urine from dosed rats was added 10 ml acetonitrile solution containing 1 mg of promazine. Each urine sample (standards and unknowns) was mixed with 0.5 ml of 0.5 M sodium carbonate and extracted with 7 ml of 15% pentane and 5% isopropanol in ethyl acetate by shaking (IKA Vibrax Shaker, Terochem Laboratories Ltd., Edmonton, Alb., Canada) for 15 min. The mixture was then centrifuged for 5 min. The organic layer obtained was dried at <45°C in a SpeedVac concentrator (Model RH 60-17 100, Savant Instruments Int., Farmingdale, NY). The residue was reconstituted into 60 μl acetonitrile and a 20 μl aliquot was injected into the conventional HPLC system. Standard curves were constructed by plotting the appropriate peak height ratio (analyte/internal standard) versus concentration of the analyte. All standards and unknowns were analyzed concurrently.

3. Results

3.1. Characterization of metabolites

A typical HPLC chromatogram of a urine extract from a rat dosed with clozapine N-oxide (Fig. 2C) gave six peaks which were not present in the chromatograms of an extract from blank urine (Fig. 2A). Three peaks had the same retention time values as those obtained from authentic clozapine (2B, **II**: 2C, peak 1), *N*-desmethylclozapine (2B, **III**; 2C, peak 5) and clozapine N-oxide (2B, **I**; 2C, peak 6). The appropriate fractions corresponding to each peak from the HPLC chromatograms were collected and analyzed by direct loop injection ESI mass spectrometry. The ESI mass spectrum obtained from the fraction corresponding to peak 6 with a retention time of 16.1 min showed the protonated molecular ion of *m/z* 343/345 as a base peak, which was identical to the spectrum of authentic clozapine N-oxide (**I**). However, the spectra obtained from all other fractions did not give enough structural information mainly due to very low quantities of the analytes and the interference of other analytes and impurities co-eluted from the conventional HPLC.

Each individual fraction was then analyzed by the more sensitive and selective ESI micro-column HPLC/MS. The analytes and non-drug related impurities were separated by the micro-column in each case. The chromatographic behaviour and ESIMS data obtained from the fractions corresponding to the above-mentioned three peaks were compared with those of authentic standards (Table 1, Figs. 3 and 4). The reconstructed ion chromatogram of the fraction of peak 1 ($R_t = 3.9$ min) showed only one peak which had a similar retention time (Fig. 4A, peak II) to that obtained from authentic clozapine (Fig. 3A, peak II). The retention times observed for peak II are not identical with those observed for the authentic refer-

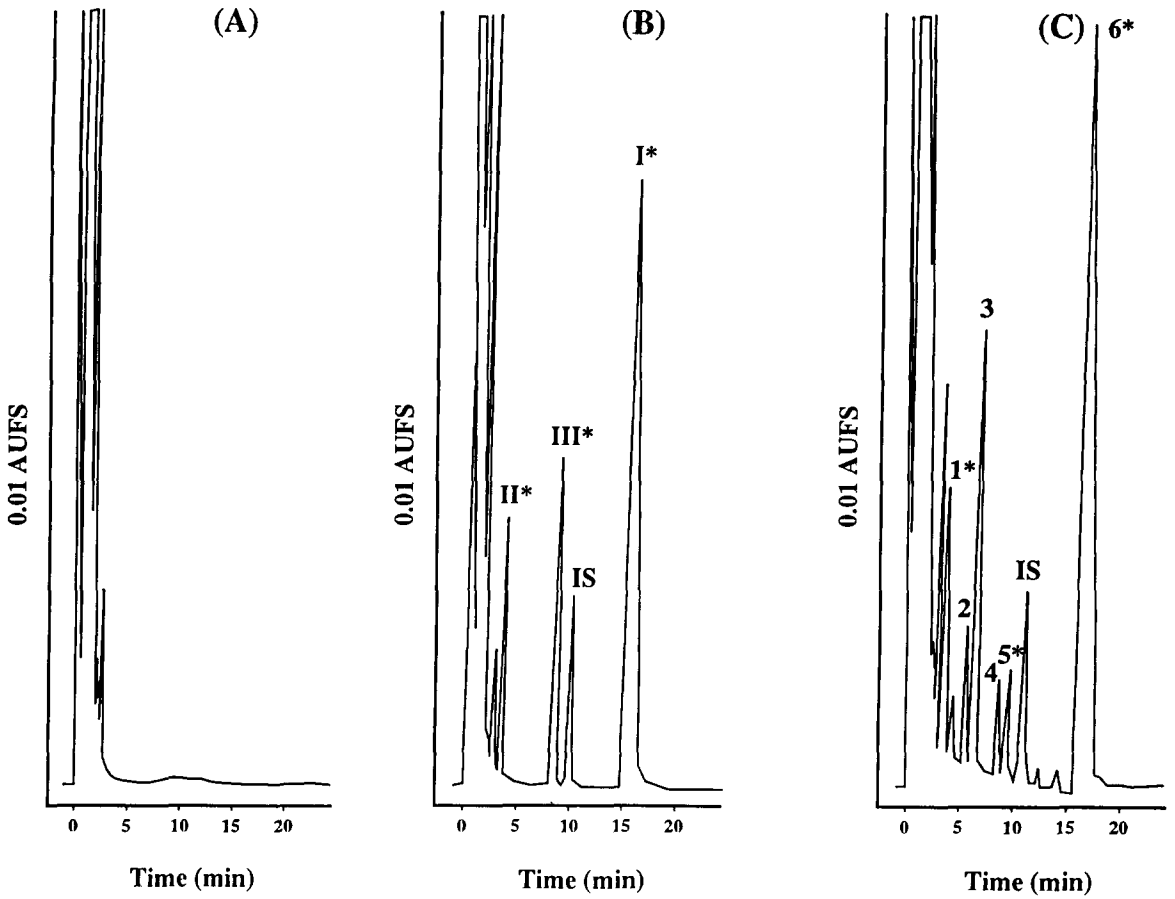


Fig. 2. Conventional HPLC chromatograms of extracts from (A) blank rat urine, (B) blank rat urine with added reference standards and (C) urine from a rat dosed with clozapine N-oxide. AUFS, absorbance units full scale. Quantified compounds are indicated by an asterisk. **I**, Clozapine N-oxide; **II**, clozapine; **III**, *N*-desmethylclozapine; IS, internal standard, promazine.

ence sample probably due to the presence of other co-extracted compounds from the biological matrix whose presence can influence the chromatographic properties during chromatography on cyano-type HPLC columns. This is especially pronounced in micro HPLC columns. The corresponding ESI mass spectra of this peak and the authentic standard were identical (Fig. 4B, spectrum II and Fig. 3B, spectrum II), and exhibited an intense protonated molecular ion of clozapine (*m/z* 327/329). Therefore, the presence of clozapine (**II**) as the metabolite of clozapine N-oxide was evident (Fig. 1). The comparison of chromatographic retention time and ESI mass spectrum of the fraction of peak 5 ($R_t = 9.0$ min) (Figs. 4A and 4B, peak III and spectrum III) with those obtained from authentic *N*-desmethylclozapine (Figs. 3A and 3B, peak III and spectrum III) indicated that this metabolite was *N*-desmethylclozapine (**III**) (Fig. 1, Table 1). Both ESI mass spectra exhibited a protonated molecular ion (*m/z* 313/315, MH^+) and a solvent adduct ion (*m/z* 343/345, $[M + 31]^+$) with high intensity. The identity of clozapine N-oxide was also confirmed by comparison of its HPLC behavior and mass spectrometric data (Figs. 4A and 4B, peak I and spectrum I) with those of an authentic standard (Figs. 3A and 3B, peak I and spectrum I, Table 1).

The reconstructed ion chromatogram analyzed by micro-column HPLC/MS for the collected fraction of peak 2 showed only one peak, the

Table 1
The identification of metabolites in organic urinary extracts of rats dosed with clozapine N-oxide using ESI micro-column HPLC/MS at a sampling cone voltage of 45 V

Compound	No.	HPLC/MS data[a]	
		R_t (min)	MH^+ (m/z)
Clozapine N-oxide	**I**	13.09/13.09	343/345 (100/31; 100/30)
Clozapine	**II**	19.14/19.16	327/329 (100/28; 100/27)
N-Desmethylclozapzine	**III**	33.55/33.52	313/315 (100/27; 45/15)
8-Deschloro-8-thiomethylclozapine	**IV**	14.91	339 (100)
N-Desmethyl-8-deschloro-8-thiomethylclozapine	**V**	14.70	325 (100)
8-Deschloro-8-hydroxy-clozapine	**VI**	21.78	309 (100)
Tert-butyldimethyl-silylated 8-deschloro-8-hydroxyclozapine	**VIa**	13.09	423 (61)
8-Deschloro-8-methyl-sulfinylclozapine	**VII**	15.10	355 (30)

[a] The retention times of the authentic compound/metabolite are shown. Mass spectral data show the appropriate protonated molecular ion (m/z) with the corresponding relative abundance (%) of authentic compound; metabolite in parentheses. Where no authentic standard was available only data for the metabolite are shown.

corresponding ESIMS spectrum with a sampling cone voltage of 45 V gave the highest mass ion of m/z 339 as a base peak with the absence of chlorine isotope ion. In order to obtain more structural fragment information, this fraction was also analyzed by micro-column HPLC/MS with a higher cone voltage (80 V). The observed diagnostic fragment ions of m/z 308, 282, 268, 252, 239, 225 and 99 indicated that the fragment pattern was in agreement with that of clozapine and that no piperazine N-oxide function was present (Table 2). The protonated molecular ion (m/z 339) corresponded to an increase of 12 mass units compared to clozapine. The absence of the typical chlorine isotope peaks indicated the loss of chlorine atom. Hence such a difference in mass can correspond to the presence of either a CH_3S group or a OH group plus a CH_3O group. The hydroxylated metabolite could be ruled out since no silylated derivative was obtained when this compound was derivatized with MTBSTFA. Therefore, this metabolite contains a CH_3S group. The thiomethyl substitution was assigned at the 8 position since this metabolite has been identified previously from patients medicated with clozapine [17] and it could be generated from the exchange of chlorine with thiomethyl group. This metabolite was then characterized as 8-deschloro-8-thiomethylclozapine (**IV**, Fig. 1).

The fraction corresponding to peak 3 collected from conventional HPLC was also analyzed by micro-column HPLC/MS with both lower (45 V) and higher (80 V) sampling cone voltages (Tables 1 and 2). The reconstructed ion chromatograms from the HPLC/MS analysis showed three peaks, although these peaks were not resolved in the total ion chromatogram (Fig. 5A). The ESI mass spectra of the reconstructed ion chromatograms gave fairly well-resolved peak profiles (Fig. 5A), in which the protonated molecular ion was the base peak for all three compounds (Fig. 5B, spectrum IV, m/z 339; spectrum V, 325; spectrum VI, 309). According to the ion current response (data not shown) the metabolite with longest retention (21.78 min, m/z 309) predominated in this fraction.

The ESI mass spectrum of the metabolite first eluting from the micro-column gave a protonated molecular ion of m/z 325 (MH^+), a natiurated molecular ion of m/z 347 (MNa^+) and a solvent adduct ion of m/z 355 ($[M+31]^+$) with no chlorine isotope peaks (Fig. 5B, spectrum V). This molecular ion corresponded to a 14 mass unit reduction compared to metabolite **IV**, 8-deschloro-8-thiomethylclozapine, which eluted second in micro-column HPLC. The diagnostic fragment pattern corresponding to the tricyclic ring ions (m/z 308, 268 and 239) observed in the

ESI mass spectrum with a higher cone voltage indicated that the substituted tricyclic moiety of this compound was the same as that of metabolite **IV** (Table 2). Therefore, this 14 mass unit reduction must be due to the demethylation of metabolite **IV** at the piperazinyl nitrogen atom. Accordingly this metabolite was assigned as *N*-desmethyl-8-deschloro-8-thiomethylclozapine (**V**, Fig. 1), which was previously reported as a metabolite of clozapine in humans [17].

The compound eluting second from the micro-column had a similar retention time (14.91 min) and an identical ESI mass spectrum (Fig. 5B, spectrum IV) to those obtained for metabolite **IV**, which was identified from the collected fraction of peak 2 analyzed by conventional HPLC. This metabolite was also detected in the fraction corresponding to peak 3 because during the conventional HPLC analysis the fraction collected as peak 3 was still contaminated with material eluting under peak 2.

The ESI mass spectrum of the last eluting metabolite from the micro-column gave a protonated molecular ion of m/z 309 and a solvent adduct ion of m/z 339 ($[M+31]^+$) with the absence of chlorine isotope ions (Fig. 5B, spectrum VI). Compared to clozapine this molecular weight corresponded to a decrease of 18 mass units, which could be predicted to be due to the presence of either an aromatic hydroxyl functional group or a piperazine N-oxide. The presence of a hydroxyl group was confirmed by a derivatization experiment. After derivatization with MTBSTFA, the retention time of the peak was reduced and the highest mass ion of m/z 423 was observed, which corresponded to the protonated molecular ion of *tert*-butyldimethylsilylated derivative (**VIa**) (Table 1). In addition, the diagnostic fragment ions of this metabolite obtained from the ESI mass spectrum at a cone voltage of 80 V showed a similar pattern to that of clozapine (Table 2). Although the position of aromatic hydroxylation could not be definitely assigned according to the present evidence, the replacement of chlorine atom with a hydroxyl group could possibly take place. The metabolite was then identified as 8-deschloro-8-hydroxyclozapine (**VI**, Fig. 1). This metabolite has also been reported previously as a metabolite of clozapine [17].

The micro-column chromatogram of the fraction of peak 4 collected from conventional HPLC yielded a single peak and the ESI mass spectra

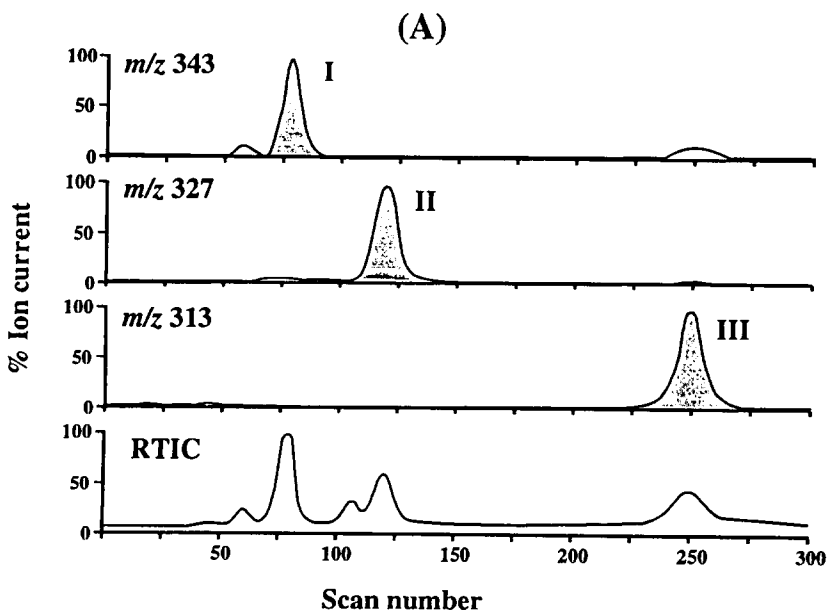


Fig 3(A).

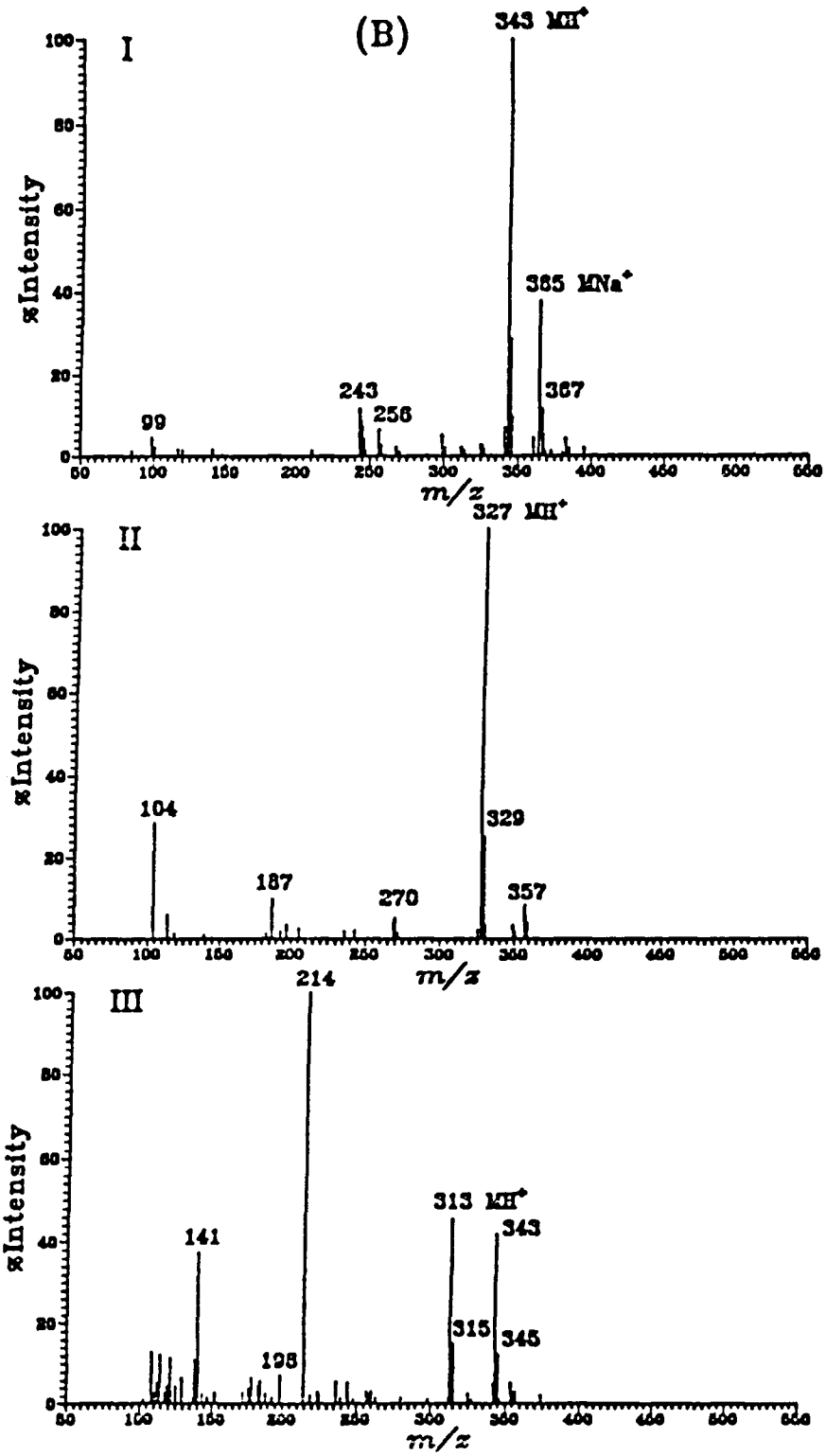


Fig. 3. ESI HPLC/MS analysis at a sampling cone voltage of 45 V for refernece standards clozapine N-oxide (**I**), clozapine (**II**) and *N*-desmethylclozapine (**III**). (A) Reconstructed total ion current (RTIC) over range of m/z 310–360 and reconstructed ion chromatograms of each corresponding analyte. (B) ESI mass spectra of each individual compound corresponding to the appropriate reconstructed ion chromatogram.

demonstrated a protonated molecular ion of *m/z* 355 at both low and high cone voltages and diagnostic fragment ions of *m/z* 308, 282, 268, 252, 239, 225 and 99 with the higher cone voltage (Table 2). The loss of the chlorine atom was indicated by the absence of chlorine isotope peaks in the ESI mass spectra. The molecular weight of this metabolite was 16 and 30 mass units higher than metabolite **IV** and metabolite **V**, respectively, and this compound could be an oxygenated metabolite **IV** or methoxylated metabolite **V**. The diagnostic fragment ion of *m/z* 99 corresponding to the methylated piperazinyl side chain confirmed the presence of methyl substituent on the piperazine, hence this metabolite might be an oxygenated metabolite **IV**. The one extra oxygen

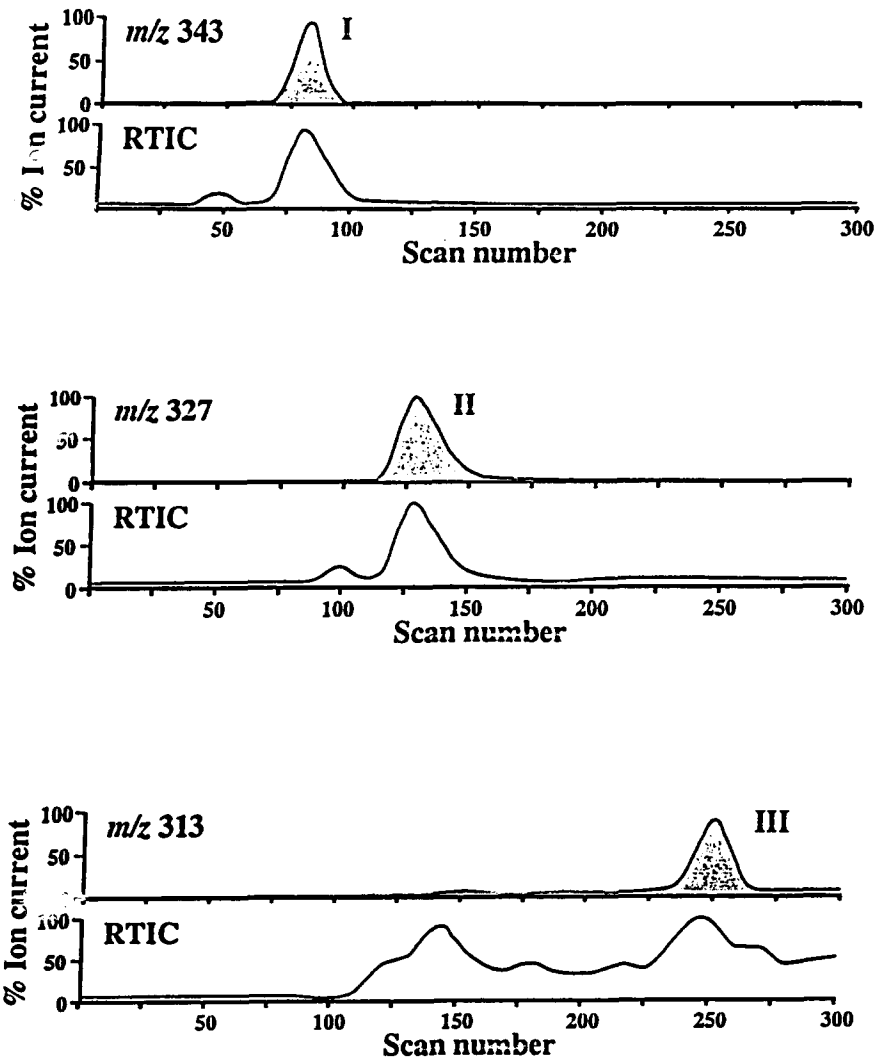


Fig. 4(A)

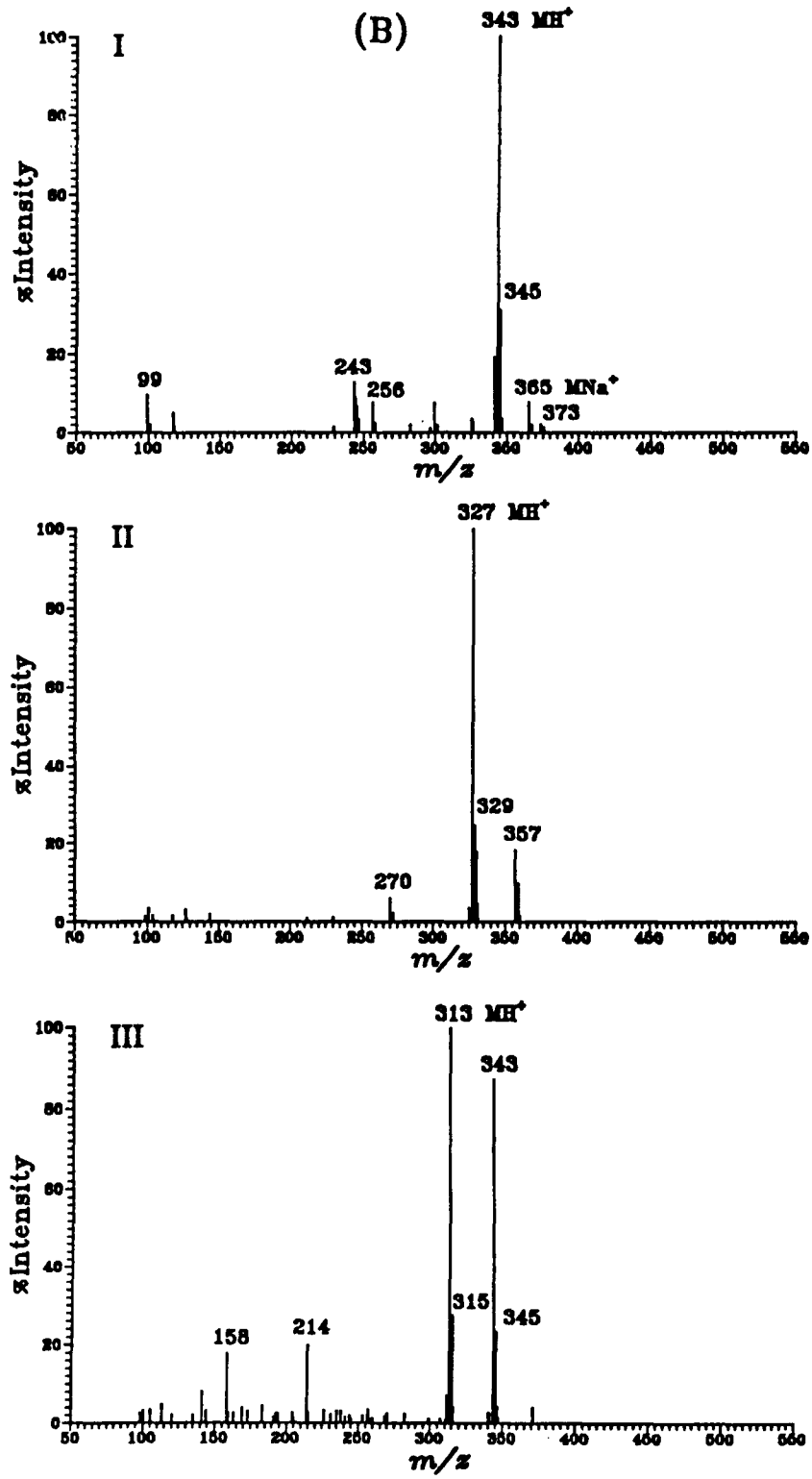
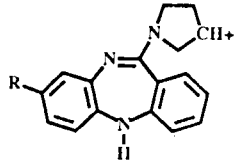
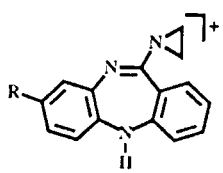
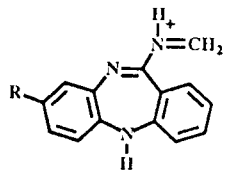
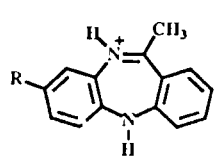
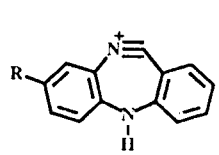
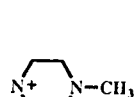


Fig. 4. ESI HPLC/MS analysis (45 V) of the fractions of peak 1 (A, peak II), peak 5 (A, peak III) and peak 6 (A, peak I) collected from conventional HPLC analysis for urinary extracts of rats dosed with clozapine N-oxide. (A) Reconstructed total ion current (RTIC, m/z 310–360) and reconstructed ion chromotograms. (B) ESI mass spectra corresponding to each appropriate reconstructed ion chromatogram. **I**, Clozapine N-oxide; **II**, clozapine; **III**, *N*-desmethylclozapine.

Table 2
The characteristic fragment ions of compounds identified in the urine of rats dosed with clozapine N-oxide by ESI micro-column HPLC/MS at sampling cone voltage of 80 V[a]

Compound (No.)	R	MH^+						
MH^+								
Clozapine N-oxide (**I**)[b]	Cl	343/345 (85/28)	296/298 (20/8)	270/272 (10/3)	256/258 (66/17)	243/245 (100/35)	227/229 (37/13)	99 (29)
Clozapine (**II**)[b]	Cl	327/329 (32/9)	296/298 (12/4)	270/272 (100/29)	NA	243/245 (10/3)	227/229 (8/3)	99 (5)
N-Desmethyl-clozapine (**III**)[b]	Cl	313/315 (100/28)	296/298 (10/5)	270/272 (60/21)	NA	243/245 (10/5)	227/229 (28/9)	*NA*
8-Deschloro-8-thiomethyl-clozapine (**IV**)	CH_3S	339 (100)	308 (14)	282 (29)	268 (17)	NA	239 (13)	99 (48)
N-Desmethyl-8-deschloro-8-thio-methylclozapine (**v**)	CH_3S	325 (100)	308 (24)	NA	268 (17)	NA	239 (2)	NA
8-Deschloro-8-hy droxyclozapine (**VI**)	HO	309 (38)	278 (9)	252 (100)	NA	225 (9)	209 (41)	99 (22)
8-Deschloro-8-methylsulfinyl-clozapine (**VII**)	CH_3OS	355 (100)	324 (15)	298 (14)	284 (64)	NA	255 (65)	NA

[a] Mass spectral data show the appropriate fragment ions (m/z) with the corresponding relative abundance (%) in parentheses. NA: Not applicable,
[b] Data for authentic reference samples.

atom substituted on metabolite **IV** could exist as a methylsulfinyl or an aromatic hydroxyl or an N-oxide group. The aromatic hydroxylation was ruled out by the silylating derivatization. The diagnostic fragment ion corresponding to the tricyclic ring ions obtained at a sampling cone voltage of 80 V (*m/z* 324, 298, 284 and 255) suggested that the substituent was a methylsulfinyl group (Table 2). Therefore, this metabolite was characterized as 8-deschloro-8-methylsulfinylclozapine (**VII**, Fig. 1).

3.2. Quantitation of clozapine N-oxide, clozapine and N-desmethylclozapine

The quantitation of clozapine N-oxide, clozapine and *N*-desmethylclozapine in rat urine was performed by an HPLC–UV procedure using an internal standard method (Fig. 2). The calibration curves for the three analytes, the ranges of these curves, their statistical relationship and other details are summarized in Table 3. The overall mean RSD for the five standard solutions over each range of the standard curve for each analyte varied between 5.3 and 10.1%. The excretions of each measured analyte from the 0–24 h urine of each rat are given in Table 4. The mean urinary excretions of clozapine N-oxide, clozapine and *N*-desmethylclozapine determined as a percentage of the dose were $0.93 \pm 0.54\%$, $0.06 \pm 0.03\%$ and $0.01 \pm 0.006\%$, respectively.

4. Discussion

ESIMS has been used extensively to study peptide and protein molecular weights [27–32]. Recently, the use of this ionization mode for small molecular weight compounds has received more attention, and the results show that the advantages of ESI reported for large molecules also apply to most, if not all, small molecular weight compounds [33–40]. The combination of this ionization mode with ODS micro-column HPLC has also been used to analyze small molecular weight compounds [38]. In general, the abundance of the protonated molecular ion (MH^+) is maximized at relatively low sampling cone voltages (30–60 V), and the intensity of structurally informative fragment ions increases with higher cone voltages.

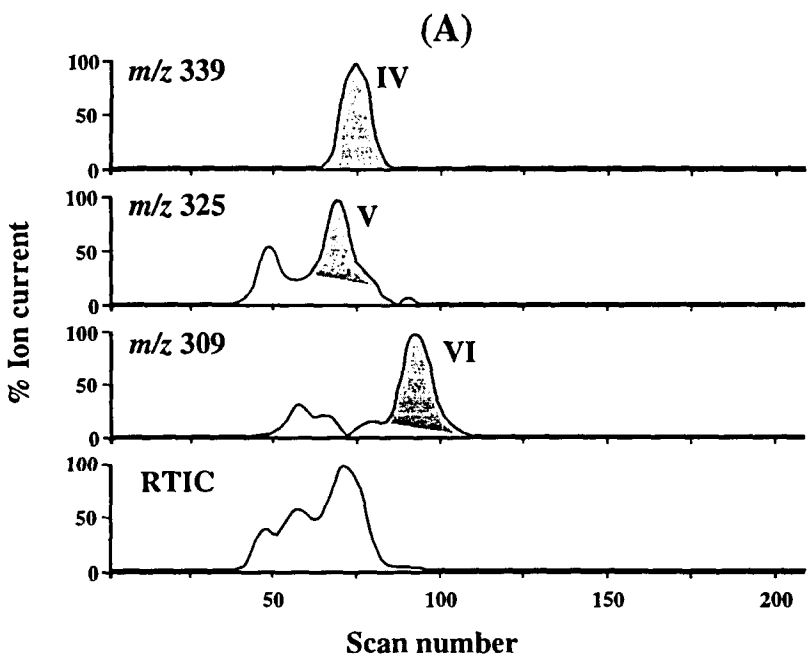


Fig 5(A)

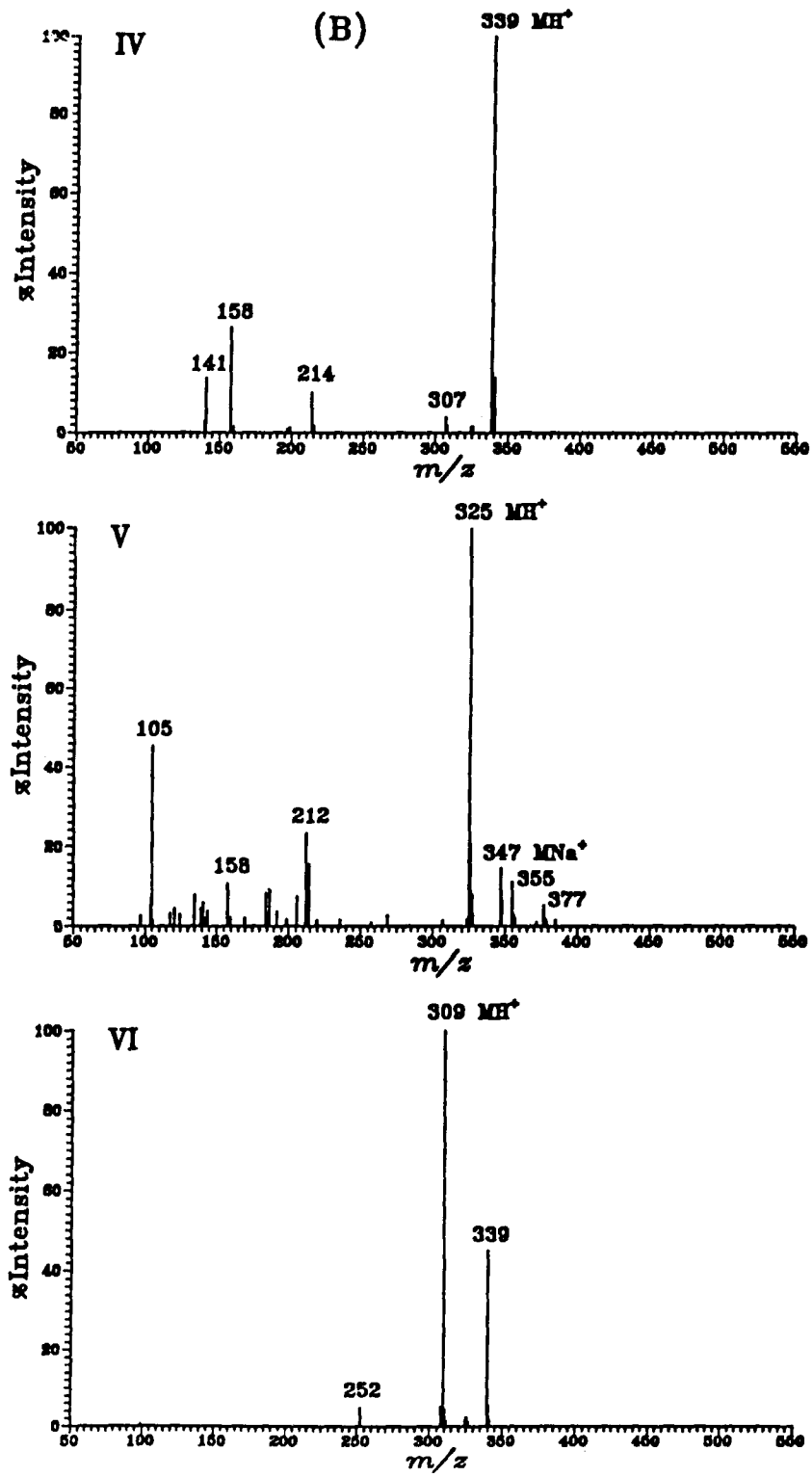


Fig. 5. ESI HPLC/MS analysis (45 V) of the fraction of peak 3 collected from conventional HPLC analysis for urinary extracts of rats dosed with clozapine N-oxide. (A) Reconstructed total ion current (RTIC, m/z 310–360) and reconstructed ion chromatograms. (B) ESI mass spectra corresponding to each appropriate reconstructed in chromatogram. **IV**, 8-deschloro-8-thiomethylclozapine; **V**, *N*-desmethyl-8-deschloro-8-thiomethylclozapine; **VI**, 8-deschloro-8-hydroxyclozapine.

Table 3
Calibration curve data for the quantitation of clozapine N-oxide, clozapine and *N*-desmethylclozapine in rat urine by conventional HPLC–UV procedures

Analyte (compound No.)	R_t (min)	Standard curve (r^2)	Concentration		
			Range[a]	Mean	%RSD[b]
Clozapine N-oxide (**I**)	16.1	$y = 0.523x + 0.285$ (1.00)	1–50 μg ml^{-1}	7.3	
Clozapine (**II**)	3.9	$y = 0.004x - 0.079$ (1.00)	0.1–1.5 μg ml^{-1}	5.3	
N-Desmethylclozapine (**III**)	9.0	$y = 0.00014x^2 + 0.002x + 0.063$ (0.99)	5–500 ng ml^{-1}	10.1	

[a] Five concentrations over each standard curve range were prepared in triplicate.
[b] The overall mean RSD of the mean values ($n = 5$) obtained at each concentration point of the standard curve range.

Thus this interface-induced decomposition can be used with advantage to obtain structural fragmention information. The present study demonstrates that on-line ESIMS with micro-column HPLC was a simple, sensitive, selective, reliable and rapid technique to analyze precisely and simultaneously various drug metabolites in extracts from biological fluids. The cyano micro-column successfully separated several components in the urinary extracts which were not clearly resolved by conventional HPLC. The observation of natiurated molecular ions (MNa^+) in the ESI mass spectra was also useful for the confirmation and identification of the molecular ions. Note also that the ESI mass spectra of most analytes showed an ion 31 mass units greater than the corresponding molecular weight (Figs. 3–5). These ions disappeared when samples were re-examined using a mobile phase with a much lower concentration of methanol (5%). Presumably these ions are generated from interaction of the analytes with methanol in the mobile phase and can also be used to confirm molecular ions.

Direct comparison with authentic reference samples confirmed the identities of clozapine N-oxide, clozapine and *N*-desmethylclozapine present in the urine of rats administered clozapine N-oxide. The identification of other metabolites was based on the ESIMS data. The aromatic substitutions of metabolites **IV, V, VI** and **VII** were all assigned at the 8-position, mainly due to the diagnostic mass fragment patterns as compared with those of structurally related authentic standards (Table 2). NMR spectroscopic studies of these metabolites are required to further confirm the position of substitution and were not successful in the present study due to the very low concentration found in the urine samples. In addition, the replacement of the chlorine atom with a hydroxyl or thiomethyl or methylsulfinyl group could be expected metabolically [17]. Metabolites **IV, V** and **VI** have been previously identified as metabolites of clozapine in patients [17].

The proposed metabolic pathway of clozapine N-oxide in rat urine is shown in Fig. 1. This paper is the first report on the metabolic profiles of clozapine N-oxide in any species and identifies for the first time 8-deschloro-8-methylsulfinylclozapine (**VII**).

5. Conclusion

In the present study, it is interesting to note that all of the metabolites identified in the urine have no N-oxide function except for the dosed clozapine N-oxide. The result may suggest that clozapine N-oxide first reduces back to the parent drug in the body, which is further converted to other metabolites. The quantitative studies demonstrate that the amount of clozapine N-oxide excreted in urine was much higher that that of the other metabolites (Table 4). Visual inspection of the appropriate peaks of the chromatograms indicates **VI** was a major metabolite and its amount was much greater than any other metabolite (Fig. 2), although it could not be quantified since no authentic standard was available. This observation suggests that unlike chlorpromazine N-oxide, which is extensively metabolized after

Table 4
The excretion (accounted as the percentage of the dose) of clozapine N-oxide, clozapine and *N*-desmethylclozapine in the 0–24 h urine of rats ($n = 6$) dosed with clozapine N-oxide

Rat No.	Sex	Clozapine N-oxide	Clozapine	*N*-Desmethylclozapine
1	Female	1.93	0.10	0.02
2	Female	1.01	0.07	0.01
3	Female	0.57	0.04	0.01
4	Male	0.96	0.10	0.01
5	Male	0.55	0.02	0.002
6	Male	0.53	0.04	0.005
Mean ± S.D.		0.93 ± 0.54	0.06 ± 0.03	0.01 ± 0.006

oral administration to rat, dog and human [10, 11], the reduction of clozapine N-oxide back to clozapine is far less extensive.

Acknowledgements

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References

[1] L. Damani, in W.B. Jakoby, J.R. Bend and J. Caldwell (Eds.). Metabolic Basis of Detoxification: Metabolism of Functional Groups. Academic Press, New York, 1982, pp. 127–149.
[2] J. Rose and N. Castagnoli, Ir., Med. Res. Rev., 3 (1983) 73–88.
[3] M.H. Bickel, H.J. Weder and M. Baggioline, Helv. Physiol. Pharmacol. Acta, 24 (1966) c77–c78.
[4] R.L.H. Heimans, M.R. Fennessy and G.A. Gaff, J. Pharm. Pharmacol., 23 (1971) 831–836.
[5] R.M. Dajani, J.W. Gorrod and A.H. Beckett, Biochem. Pharmacol., 24 (1975) 109–117.
[6] R.E. McMahon and H.R. Sullivan, Xenobiotica, 7 (1977) 377–382.
[7] P. Jenner, J.W. Gorrod and A.H. Beckett, Xenobiotica, 3 (1973) 341–349.
[8] G. Powis, M.M. Ames and J.S. Kovach, Cancer Res., 39 (1979) 3564–3570.
[9] M. Al-Walz, R. Ayeshi, S.C. Mitchell, J.R. Idle and R.L. Smith, Clin. Pharmacol. Ther., 42 (1987) 608–612.
[10] T.J. Jaworski, E.M. Hawes, G. McKay and K.K. Midha, Xenobiotica, 18 (1988) 1439–1447.
[11] T.J. Jaworski, E.M. Hawes, G. McKay and K.K. Midha, Xenobiotica, 20 (1990) 107–115.
[12] T.J. Jaworski, E.M. Hawes, J.W. Hubbard, G. McKay and K.K. Midha, Xenobiotica, 21 (1991) 1451–1459.
[13] A. Nagy and T. Hansen, Acta Pharmacol. Toxicol., 42 (1978) 58–67.
[14] H. Koch, Drugs Today, 18 (1982) 109–115.
[15] M.H. Lewis, E. Widerlov, D.L. Knight, C.D. Kilts and R.B. Mailman, J. Pharmacol. Exp. Ther., 225 (1983) 539–545.
[16] R. Gauch and W. Michaelis, Farmaco, 26 (1971) 667–681.
[17] V.B. Stock, G. Spiteller and R. Hetpertz, Arzneim.-Forsch. Drug Res., 27 (1977) 982–990.
[18] V. Fischer, B. Vogels, G. Maurer and R.E. Tynes, J. Pharmacol. Exp. Ther., 260 (1992) 1355–1360.
[19] U. Breyer and K. Villumsen, Eur. J. Clin. Pharmacol., 9 (1976) 457–465.
[20] M. Ackenhell, Psychopharmacol., 99 (1989) S32–S37.
[21] M.G. Choc, R.G. Lehr, F. Hsuan, G. Honigfeld, H.T. Smith, R. Borison and J. Volavka, Pharm. Res., 4 (1987) 402–405.
[22] Y.F. Cheng, T. Lundberg, U. Bondesson, L. Lindstrom and J. Gabrielsson, Eur. J. Clin. Pharmacol., 34 (1988) 445–449.
[23] M.G. Choc, F. Hsuan, G. Honigfeld, W.T. Robinson, L. Ereshefsky, M.L. Crimson, S.R. Saklad, J. Hirshowitz and R. Wagne, Pharm. Res., 7 (1990) 347–351.
[24] U. Bondesson and L.H. Lindstrom, Psychopharmacol., 95 (1988) 472–475.
[25] P.J. Perry, D.D. Miller, S.V. Arndy and R.J. Cadoret, Am. J. Psychiatry, 148 (1991) 231–235.
[26] J. Meier, Br. J. Pharmacol., 53 (1975) 440.
[27] C.M. Whitehouse, R.N. Dreyer, M. Yanashita and J.B. Fenn, Anal. Chem., 57 (1985) 675–679.
[28] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong and C.M. Whitehouse, Science, 246 (1989) 64–71.
[29] J.A. Loo, H.R. Udseth and R.D. Smith, Anal. Biochem., 179 (1989) 404–412.
[30] S.K. Chowdhury, V. Katta and B.T. Chait, Rapid Commun. Mass Spectrom., 4 (1990) 81–87.
[31] R.D. Smith, J.A. Loo, C.G. Edmonds, C.J. Barinaga and H.R. Udeseth, Anal. Chem., 62 (1990) 882–899.

[32] P. Thibault, C. Paris and S. Pleasance, Rapid Commun. Mass Spectrom., 5 (1991) 484–490.
[33] W.A. Korfmacher, M.P. Chiarelli, J.O. Lay, J. Bloom, M. Holcom and K.T. McManus, Rapid Commun. Mass Spectrom., 5 (1991) 463–468.
[34] G. K. Poon, P. Mistry and S. Lewis, Biol. Mass Spectrom., 20 (1991) 687–692.
[35] S.T. Weintraub, R.N. Pinckard and M. Hail, Rapid Commun. Mass Spectrom., 5 (1991) 309–311.
[36] S. Akashi, U. Niitsu, R. Yuju, H. Ide and K. Hirayama, Biol. Mass Spectrom., 22 (1993) 124–132.
[37] H.B. Hines, Biol. Mass Spectrom., 22 (1993) 243–246.
[38] G.K. Poon and L.J. Griggs, J. Chromatogr., 628 (1993) 215–233.
[39] E.C. Huang, T. Wachs, J.J. Conboy and J.D. Henton, Anal. Chem., 62 (1990) 713a–724a.
[40] I.M. Johansson, R. Pavelka and J.D. Henion, J. Chromatogr., 559 (1992) 515–528.