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On-line nonaqueous capillary electrophoresis and electrospray mass spectrometry of tricyclic antidepressants and metabolic profiling of amitriptyline by *Cunninghamella elegans*

An on-line nonaqueous capillary electrophoresis-electrospray mass spectrometry (ESI-MS) technique was developed using a commercial ion spray interface. The nonaqueous capillary electrophoresis ESI-MS system was used to profile tricyclic antidepressants of similar structures and mass-to-charge ratios. We found that pure methanol can be used as a sheath liquid to obtain stable ion spray from nonaqueous capillary electrophoresis. The flow rate of the coaxial nebulizing gas affected baseline signals, separation efficiency, and migration times. Other nonaqueous capillary electrophoresis operating conditions and electrospray parameters were optimized for enhanced baseline separation and high sensitivity detection. The effect of sample stacking on separation and detection was evaluated. The calculated detection limits were approximately 3 pg injected onto the capillary. ESI mass spectra of tricyclic antidepressants from a single quadrupole MS were obtained and elucidated. The information was used to propose fragmentation pathways of the tricyclic antidepressants. The method was also used to analyze the metabolites of amitriptyline produced by the fungus *Cunninghamella elegans*. Sixteen metabolites were detected and most of them were tentatively identified as demethylated and/or hydroxylated, and/or *N*-oxidized products.

1 Introduction

We reported the high sensitivity analysis of five tricyclic antidepressants by nonaqueous capillary electrophoresis and thermo-optical absorbance detection in a paper in this issue [1]. Although the thermo-optical detector provides high sensitivity, it does not provide sufficient information to allow structural elucidation of metabolites. In contrast, electrospray ionization-mass spectrometry (ESI-MS) provides spectrum rich in information that may be useful for metabolite identification. Since 1990, the application of CE-ESI-MS has been widespread [2–7]. The technique, however, is still in its infancy and has some limitations, including poor sensitivity for neutral molecules such as cholesterol and aromatic hydrocarbons. Poor compatibility of CE running buffers with ESI-MS is another important limitation. Commonly used aqueous separation systems are not suitable for coupling directly to ESI-MS due to the presence of nonvolatile salts, beta-cyclodextrins, or surfactant additives [8–10]. These compounds inhibit ionization even though nebulization is unaffected, leading to very low sensitivity, or they can affect both tertiary and quaternary structures of proteins under conditions used for ESI-MS, thereby complicating analysis [11–13].

Since 1993, the use of nonaqueous buffer solutions for the separation of pharmaceuticals has been reported [14–20]. A very high separation selectivity was obtained in a nonaqueous CE system without adding surfactants or complexing agents to the electrophoresis media. The totally organic system with a volatile electrolyte is ideal for coupling to an electrospray ionization mass spectrometer. A few reports have documented the application of on-line CE-MS analysis using nonaqueous solvents [9, 15]. Tomlinson *et al.* [14, 15] discussed the use of nonaqueous separation conditions for the investigation of drug metabolites by on-line CE-MS. Lu *et al.* [11] reported a nonaqueous, surfactant-containing media CE system with on-line coupling to ESI-MS for the analysis of tamoxifen and its metabolites. We present the first report of on-line CE-MS analysis of tricyclic antidepressants.

2 Materials and methods

2.1 Materials and chemicals

Desipramine (DES), nontriptyline (NOR), imipramine (IMI), amitriptyline (AMI) and clomipramine (CLO) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were purchased from BDH (Toronto, ON, Canada). Ammonium acetate was obtrained from Anachemia (Montreal, PQ, Canada). All stock solutions (10.0 mM) were prepared by dissolving the antidepressants directly into methanol-acetonitrile (1:1). They were then diluted to 0.1–100 μM concentration with the CE running buffers or diluted running buffers before use. Typically, a 100 mM stock solution of nonaqueous electrolyte medium was prepared by dissolving ammonium acetate in methanol, then a portion of this solution was diluted to a proper concentration with methanol and acetonitrile before use. For example, 50 mM NH_4Ac in methanol-acetonitrile (75:25) was used as a running buffer, and 5 mM NH_4Ac in methanol-acetonitrile (75:25) was the sample matrix.

2.2 Culture incubation and sample preparation

Six cultures of *Cunninghamella elegans* were prepared and incubated for ten days following the same procedures and

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Abbreviations: **IS**, ion spray voltage; **OR**, orifice voltage; **SIM**, selective ion monitoring; **TIE**, total ion electropherogram

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under the same conditions described elsewhere [1, 21]. Three of these cultures were dosed with 5 mg and the rest were dosed with 10 mg of AMI. Two controls, one containing the growth medium and cell culture without AMI, and another containing only growth medium, were also prepared and incubated under the same conditions. The dry extracts were prepared as in the preceeding paper and stored at 4 °C prior to use [1]. The dried extracts were concentrated by redissolving the sample in 1 mL of methanol. A 25 μL aliquot of the 10 mg dosed sample and a 50 μL aliquot of the 5 mg dosed sample was diluted to 1 mL in a methanol-acetonitrile (75:25) solution containing 5 mM ammonium acetate. These concentrated solutions contained approximately 0.9 mM of total analyte. Control samples, without AMI, and blank samples, without AMI or cells but containing only media, were prepared in the same way. Aqueous solutions of AMI were prepared by dissolving 5 or 10 mg in 50 mL of methanol-water. A 1.5 mL aliquot of the mixture was dried with a vacuum concentrator (SpeedVac, Savant, Farmindale, NY) for 5 h and then redissolved in 0.15 mL methanol. A solution containing 0.9 mM AMI was prepared in a methanol acetonitrile (75:25) solution containing 5 mM ammonium acetate. Aqueous cell extract solutions were also injected directly without concentration or redissolution in organic solvent.

2.3 CE

CE was performed using a SpectraPhoresis 100 system from Thermo Separation Products (San Jose, CA, USA). Untreated fused-silica capillaries (Polymicro Technolocies, Phoenix, AZ, USA) with 80 cm × 50 μm ID × 184 μm OD were used for separations. Fresh running buffers were used to flush the capillaries before and between runs. Samples were introduced into the capillaries by electrokinetic injections at 10 kV for 10 s unless otherwise indicated. The inlet and outlet of the capillary were adjusted to the same height. The separations were performed at 25 kV with a current of 17 μA. The operating electrolyte was changed every 5 to 10 runs to avoid contamination and solvent evaporation.

2.4 ESI-MS

ESI-MS of CE eluents was conducted on-line with a PE/Sciex API 100 (Thornhill, ON, Canada) single quadrupole mass spectrometer equipped with an ion spray source. The CE column was inserted 0.5–1.0 mm inside the electrode tube tip, while the electrode tube was 0.5–1.0 mm outside the nebulizer tube. The coaxial sheath liquid of 100% methanol was pumped by a Harvard Apparatus low pressure syringe infusion pump (South Natick, MA, USA) at a flow rate of 2.0 μL/min through a capillary of 50 μm ID × 184 μm OD connected to the hold-down tee of the ion sprayer, and to the electrode tube surrounding the CE separation column. Before CE-MS analysis, the position of the ion sprayer was adjusted for maximum sensitivity while analytes were infused through the CE column at a rate of 2.0 μL/min. Positive ion mode was used for all measurements. Other operating parameters such as ion spray voltage (IS), orifice voltage (OR), ring voltage (RNG) and curtain gas flow were optimized for maximum total ion electrophoretic signals over a range of *m/z* 50–325 using the same flow injection method. Lower OR was chosen to generate less in-source collision-induced dissociation to monitor the protonated molecular ions. A working pressure of 1.7×10^{-5} Torr was maintained in the analyzer chamber and the ion detector (CEM) voltage of 2.3 kV was kept constant during routine operation of the instrument. When CE separation was performed, the IS was set to 4.6 kV, OR was 45 V, RNG was 320 V, curtain gas flow was 8 (0.95 L/min). Nebulizing assistance was provided by passing prepurified nitrogen through the nebulizer tube at a rate of 0.41 L/min (NEB 3) or 0.67 L/min (NEB 4), unless otherwise indicated. During sample injection the IS was set to 0 and the nebulizer gas flow was set to 0.03 L/min (NEB 0). Full scan analysis of sample ions was accomplished by scanning the quadrupole in 1.0 amu increments form 50–325 amu in 0.56 s. For the metabolite study, the scan range was *m/z* 50–650 amu. While screening for glucuronide conjugates, the enlarged range was used, the step size was set to 1.0 amu, dwell time was 1.5 ms, and the speed was 0.9 s/scan. For selective ion monitoring (SIM), ions *m/z* 72, 86, 191, 208, 233, 264, 267, 278, 281, 315 and 317 were selected, the step size was 0.2 amu, the dwell time was 5.0 ms, and the speed was 0.52 s/scan. CE-MS electrophergorams were acquired by a LCTune software, and then processed by Multiview 1.2 and MacQuant software from Sciex.

3 Results and discussion

3.1 Optimization of ESI

A mixture of 10 μM each of tricyclic amines dissolved in an acetic acid solution methanol-H_2O-acetic acid, 50:50:0.2 v/v/v), in methanol, in acetonitrile and in mixed methanol-acetonitrile solutions were infused using the syringe pump. Except for the acetic acid mixture, all solvents gave good electrospray signals for these analytes. When CE-MS mode was performed, and when a mixture of analytes was continuously injected electrokinetically *via* the CE capillary, the effect of different sheath solutions on ESI senitivity of analytes was evaluated. The acetic acid solution, methanol, acetonitrile, and mixed methanol-acetonitrile solvents were investigated. The highest signals and most stable baselines were obtained by using methanol as the sheath liquid.

An effect of sheath flow rate on the total ion electropherogram (TIE) response of the amines migrating from the CE column was also investigated. By changing the sheath flow from 5 μL/min to 2 μL/min, the peak area and height for nortriptyline and amtriptyline both increased by a factor of 1.4 without affecting the CE resolution or efficiency. The baseline became unacceptable noisy once this rate was lowered to less than 2 μL/min.

In addition to the sheath liquid, a coaxial nitrogen gas flow was employed to pneumatically assist the electrospray process. This nebulizing gas did not interfere with the ESI process, but did substantially enhance nebulization efficiency. For example, large droplets were quite visible when IS voltage was off and nebulizing gas flow was less than 0.67 L/min (NEB 4). Above 0.67 L/min, the spray became focused and a finely divided aerosol was formed. Furthermore, a significant change in CE resolution was

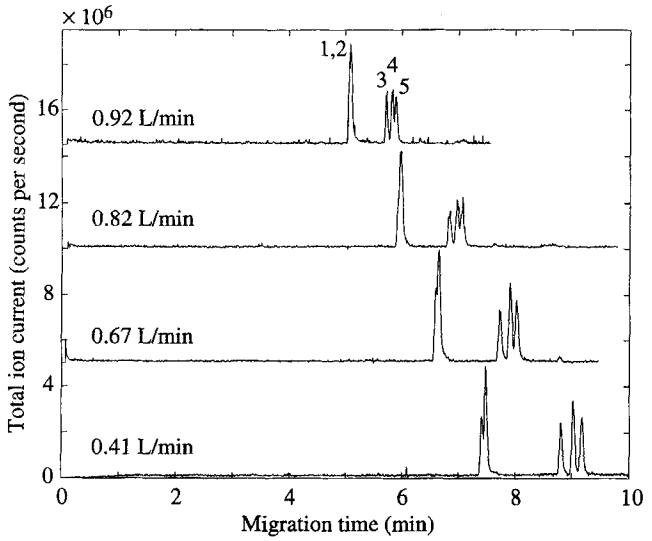

Figure 1. TIEs (baseline subtracted) showing the effect of nebulizing gas flow rate on separation and migration times of five tricyclic amines. The sample mixture was dissolved in 5 mM ammonium acetate buffer prepared in methanol-acetonitrile (60:40). The separation was performed at 25 kV, buffer was 50 mM ammonium acetate in methanol-acetonitrile, (60:40), and the current was 18 μA. Sheath fluid was methanol at a rate of 2 μL/min. Peak order: 1, desipramine; 2, nortryptyline; 3, imipramine; 4, amitryptyline; 5, clomipramine.

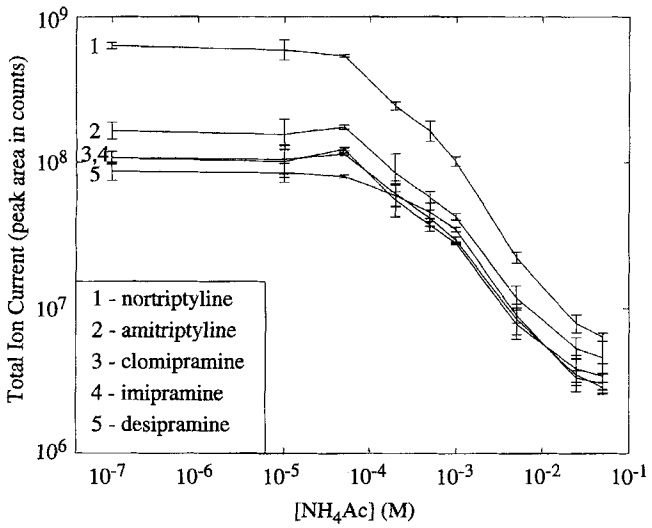

Figure 2. Effect of ammonium acetate concentration in the sample solution on total ion current. The sample mixture was dissolved in ammonium acetate buffers prepared in methanol-acetonitrile (80:20). The running buffer was 50 mM ammonium acetate in methanol-acetonitrile (80:20). The data are plotted on a log-log graph. Propagation of errors was used to plot the data at ± one standard deviation. The lines are used to connect the data points as a guide to the eye.

observed by varying the gas flow rate as illustrated in Fig. 1. Increasing the rate from 0.17 L/min (NEB 1) to 0.41 L/min (NEB 3) had little influence on CE resolution. However, when this rate was increased to 0.67 L/min (NEB 4) and above, the separation became worse and the migration times of analytes were reduced. The change in migration time could be explained by the pressure difference between inlet and outlet of the CE column. A high gas flow increases the pressure difference across the capillary, which generates more pressure-driven flow in the capillary in addition to electroosmotic flow. This result suggested the use of nebulizingflow rates in the range of 0.17 L/min to 0.41 L/min (NEB 1 to NEB 3) for a better separation. Moreover, this rate should be reduced to 0 during the sample injection procedure to avoid siphoning. However, the TIE baseline was also affected by the flow rate of nebulizing gas; at a lower rate (*e.g.* 0.26 L/min, NEB 2), large spikes in the TIE were occasionally observed, probably due to large charged droplets passing into the mass analyzer. In principle, migration times will decrease at very high nebulizing flow rates; however, we did not experience this phenomenon at the flow rates employed. Migration times were very reproducible. The average migration time of AMI was 9.87 min with a relative standard deviation of 0.7% (n = 12). Peak area was also reproducible, with an 8% relative standard deviation for CE-ESI-MS detection of AMI (n = 5).

3.2 Sample stacking effect, quantitation calibration curve and detection limits

Stacking has been used to increase sensitivity in CE [22]. In order to evaluate the loading capabilities of the CE-ESI-MS, sample stacking was studied by injecting several sample solutions that varied in the electrolyte concentration (Fig. 2). An increase in the ionic strength of the sample buffer had little effect for concentrations of ammonium acetate below 10^{-4} M. Other ions in the solvent presumably dominated the ionic strength of the solution. Higher ammonium acetate concentration resulted in lower signal. Neither migration time nor resolution were effected by stacking for anlayte concentration below 10 μM. Injection in electrophoresis is a coloumetric process, wherein passage of charge during injection leads to introduction of a proportional amount of ions. If the analyte ions constitute a large fraction of the total inionic strength of the solution, then they will make up a corresponding fraction of the ions that are injected onto the capillary. The slope of the log signal *versus* log ammonium acetate concentration plot was –0.5 for ammonium acetate concentrations above 10^{-4} and for all analyte. This slope reflects the low dissociation constant of ammonium acetate in the organic solvent; a square-root dependence is expected for the ionic strength as a function of total salt concentration. CE-ESI-MS calibration curves were prepared for samples in 5 mM NH_4Ac, a separation buffer of 50 mM ammonium acetate, 10 kV injection for 10 s, and full scan mode detection. The calibration curves were linear over an analyte concentration range of 1 μM – 50 μM; the average X^2_v value was 0.74 for four degrees of freedom, p = 0.55. The curve became convex due to peak-tailing at a concentration of 100 μM and above. In full scan mode, the concentration detection limits (3σ) were 0.5 μM for desipramine and nortryptyline and 1 μM for imipramine, amitriptyline and clomipramine, respectively. While in SIM mode, detection limits were 0.1 μM and 0.2 μM, respectively. Considering the sample stacking effect, the calculated detection limits of mass for amitriptyline were 13 pg in full scan mode and 2.5 pg in SIM mode, respectively.

3.3 ESI-MS characterization of tricyclic antidepressants

The *N*-linked structures of tricyclic antidepressants were detected with the highest sensitivity in the positive-ion

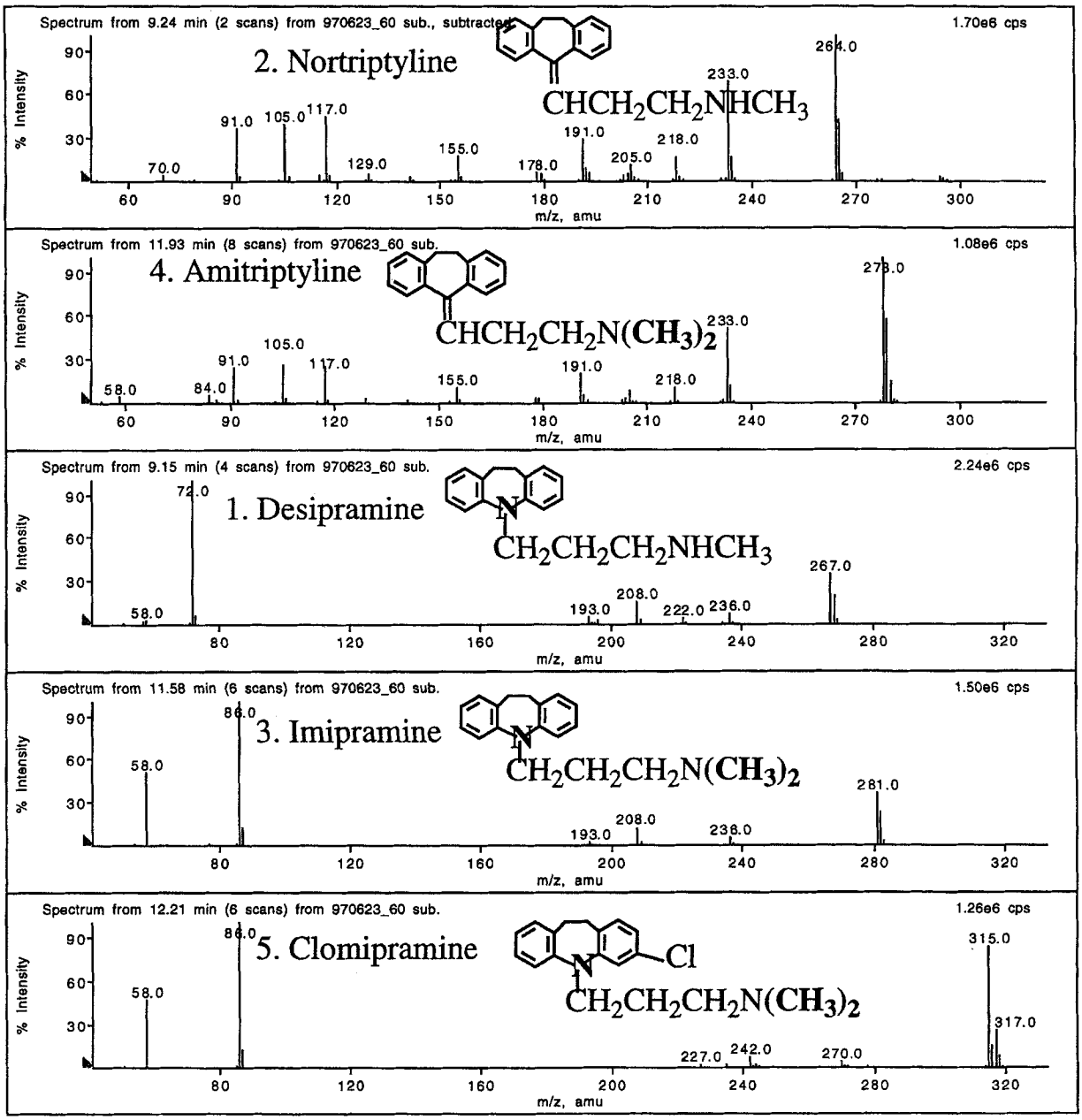

Figure 3. ESI-MS spectra of five tricyclic amines. Nebulizing gas was set to 0.41 L/min (NEB 3). The separation buffer was 50 mM ammonium acetate in methanol-acetonitrile (85:15). Other condition are the same as Fig. 1.

detection mode. Protonated antidepressants were generated in appreciable abundance even when the collision-induced dissociation (CID) potential difference between the orifice and skimmer (OR) was increased to 200 V. It was found that higher CID voltage generated more fragmentation at the expense of lower TIE signals. Moreover, the fragmentation of protonated parent ions still occurred even when the CID voltage was set to a minimum value. In this study, CID voltage was adjusted to 45 V, under which the highest TIE response, abundant protonated molecular ions, and some fragment ions were generated (Fig. 3). Figure 4 shows possible fragmentation pathways of those amines. The ESI-MS spectra of desipramine, imipramine and clomipramine were relatively simple. Bond cleavages rarely occurred on the tricyclic moiety and fragment ions were mainly due to the side chain breakage. Nortriptyline and amitriptyline showed similar spectra, except for a mass difference of 14 between their protonated molecular ions. In addition to the ions from side chain cleavages, there were also dominant cation peaks at *m/z* 91, 105, 117 and 155, reflecting the existence of bond cleavage on the tricyclic moiety.

3.4 CE-MS profiles of amitriptyline and its metabolites

Figure 5 shows the TIEs of a standard mixture of five antidepressants, an extract from 5 mg dosed *C. elegans* culture solution, and an extract from 10 mg dosed culture solution. Aqueous culture solutions were either injected directly without further clean-up or with the sample

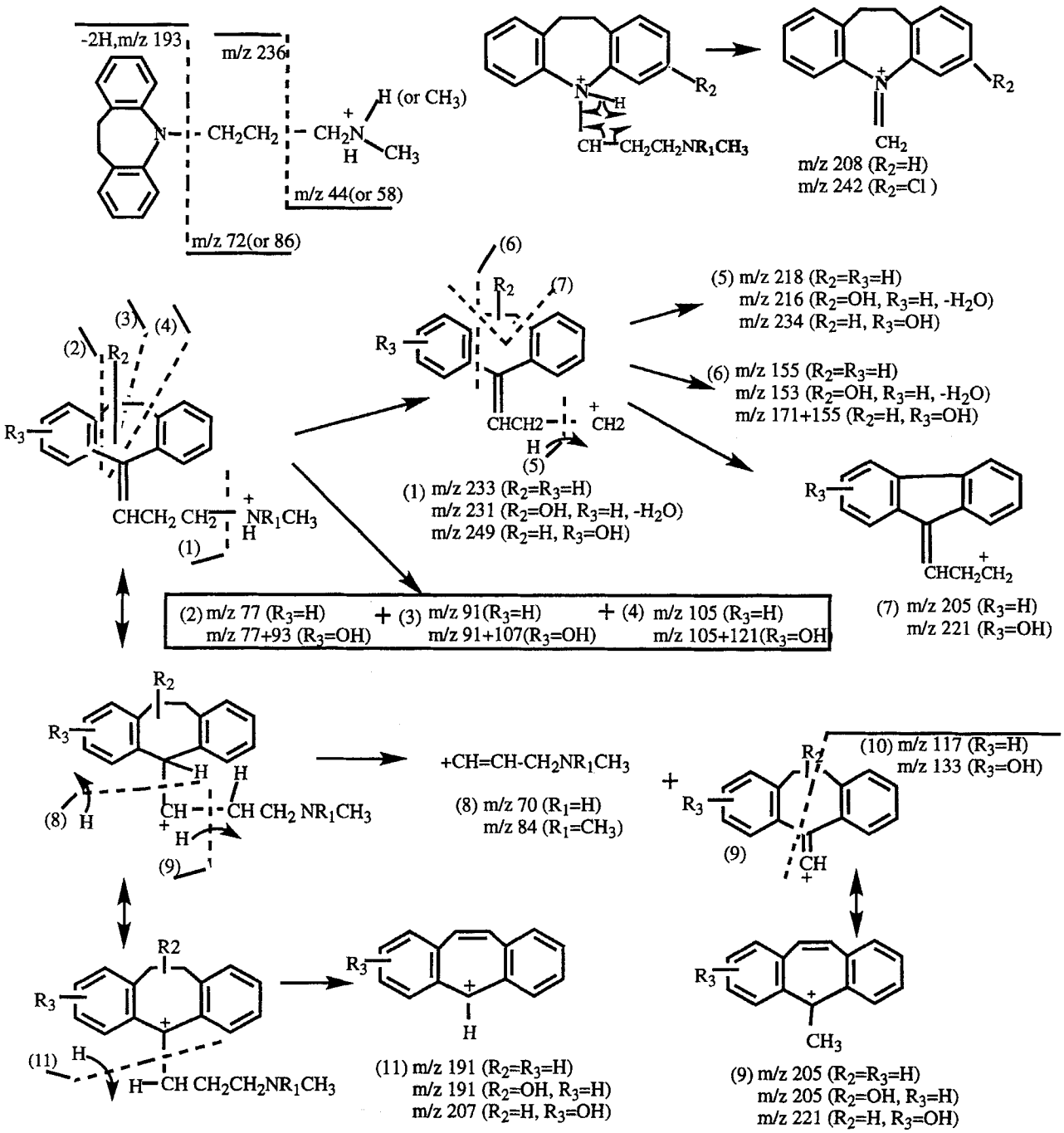

Figure 4. Proposed ESI-MS fragmentation pathways of tricyclic antidepressants and metabolites.

preparation steps. All sample solutions (extracts, vacuum-dried aqueous cultures, original aqueous cultures) gave similar profiles of metabolites except for different TIE intensities due to different dilution factors. There were few differences in the profiles of the two extracts. In addition to the parent drug, amitriptyline, 16 new compounds were detected in both extracts that were not observed in the control and blank samples. The CE-ESI-MS data are summarized in Table 1. Based on the comparison of migration times with standards and the interpretation of their spectra, the metabolites could be tentively identified as demethylated and/or hydroxylated products of amitripyt-line. Peak 3, for example, was confirmed as a demethylated metabolite, nortriptyline, by comparing its migration time and spectrum with NOR standard, and by a mass loss of 14 in protonated molecular ion compared to amitriptyline.

Peak 11 was identified as a hydroxylated product of AMI by investigating its abundant protonated molecular ion at *m/z* 294. The mass spectrum did not permit us to specify the exact position of the hydroxyl group; however, it could be localized in the tricylic moiety by the appearance of peaks at *m/z* 58 and 84 of the tertiary amines. Furthermore, the existence of a base peak at *m/z* 276 indicated that a loss of

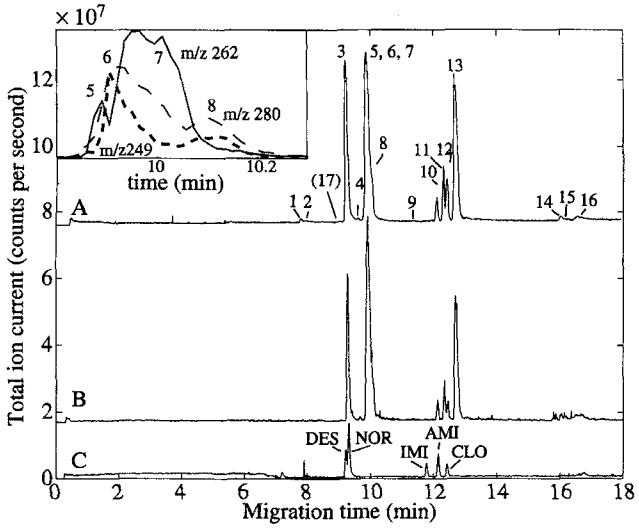

Figure 5. CE-ESI-MS profiles of AMI and its metabolites. The separation was performed at 25 kV in a 50 mM ammonium acetate buffer preparaed in methanol-acetonitrile (85:15). The current was 17 μA. Samples dissolved in 5 mM ammonium acetate buffer prepared in methanol-acetonitrile (85:15). Injection was performed at 20 kV for 10 s at a current of 13 μA. The nebulizing gas was set at 0.26 L/min (NEB 2). For capillary conditions, see Fig. 1. The peak identification is as in Table 1. The insert is the selected ion electropherogram at the indicated masses. (A) 10 mg dosed extracts; (B) 5 mg dosed extracts; (C) 10 μM mixture of five antidepressants.

H_2O from the protonated molecular ion was facile, reflecting the fact that the hydroxyl group was on either carbon 10 or 11. The appearance of peaks at *m/z* 231, 216, 205, 191 and 153, which were well elucidated from the fragmentation patterns, also support this assumption. Peak 9 gave a similar spectrum to peak 11 but more ions existed. Its structure could not be confirmed because of its low concentration. Peak 12, on the other hand, represented another kind of hydroxylated metabolite. Its spectrum indicated that it was not a 10- or 11-OH-amitryptyline due to the absence of *m/z* 276. However, the presence of *m/z* 249 suggested that the -OH was on the aromatic ring, and could be on the 2- or 3- carbon. Ions at *m/z* 234, 221, 207, 155 plus 171, and 133 supported this conclusion. Peaks 5, 6, 7 and 8 comigrated on the basis of their SIM profiles (insert in Fig. 5). They were characterized by examining either *m/z* 262 $[M+H]^+ - 18$) or *m/z* 249 ($[M+H]^+ - HNR_1CH_3 + 16$), and by examining either *m/z* 231, 216, 153 or *m/z* 234, 221, 155. Peak 4 and peak 14 may be *N*-oxidized poducts because of the presence of ($[M+H]^+ - 16$) and ($[M+H]^+ - 61$) and the absence of *m/z* 84 plus 58, or *m/z* 70 plus 44, and the similarity of other fragments to nortryptyline (peak 4 *vs.* peak 3) or to amitryptyline (peak 14 *vs.* peak 10). As for the di-OH-amitryptyline, peak 15, the precise positions of two OH- groups have not been determined. Mass spectra of peaks 1, 2 and 16 were related to that of the above-mentioned metabolites, but their identification needs further investigation. Peak 17 appeared only in the profiles of much

Table 1. CE-ESI-MS data of amitriptyline and its metabolites

Peak No.	Compound	MT (min)	$[M+H]^+$ (%)	Characteristic ions (%)
1	Met-1	7.88	?	342(34), 308(6), 294(5), 276(13), 249(6), 231(100), 216(65), 153(3), 58(30)
2	Met-2	8.04	?	342(76), 308(20), 294(69), 276(18), 249(6), 231(100), 221(35), 216(77), 207(28), 153(3), 107(28), 58(41)
3	Nortriptyline(NOR)	9.27	264(100)	233(96), 218(36), 205(26), 191(57), 178(15), 155(33), 117(68), 105(66), 91(60), 70(10)
4	*N*-oxide-NOR	9.72	280(45)	264(89), 233(27), 219(100), 191(23), 155(9), 117(24), 105(31), 91(10), 44(54)
5	11(or 10)-OH-NOR	9.87	280(29)	262(93), 231(100), 216(86), 205(16), 191(38), 153(11), 133(5), 115(6), 70(11), 44(8)
6	2(or 3)-OH-NOR	9.92	280(83)	249(86), 234(31), 221(26), 207(46), 171(13), 155(41), 133(33), 121(57), 117(24), 107(57), 105(40), 91(23), 70(4), 44(4)
7	10(or 11)-OH-NOR	10.02	280(26)	262(93), 231(100), 216(93), 205(23), 191(44), 153(15), 115(10), 70(15), 44(17)
8	3(or 2)-OH-NOR	10.13	280(100)	249(71), 234(18), 221(18), 207(52), 171(7), 155(18), 133(25), 121(29), 117(14), 107(34), 105(21), 91(8), 70(17), 44(3)
9	Met-9	11.44	294(34)	276(100), 231(14), 217(8), 215(16), 185(34), 153(22), 105(5), 84(33), 58(61)
10	Amitriptyline(AMI)	12.19	278(100)	233(67), 218(18), 205(13), 191(30), 178(7), 155(16), 117(45), 105(42), 91(33), 84(10), 58(5)
11	10(or 11)-OH-AMI	12.37	294(48)	276(100), 231(86), 216(64), 205(23), 191(27), 153(8), 117(8), 115(7), 107(4), 91(4), 84(15), 58(48)
12	2(or 3)-OH-AMI	12.51	294(100)	249(64), 234(15), 221(13), 207(21), 171(5), 155(18), 133(17), 121(26), 117(11), 107(24), 105(18), 91(10), 84(6), 58(8)
13	11(or 10)-OH-AMI	12.78	294(57)	276(100), 231(94), 216(78), 205(31), 191(35), 153(12), 133(3), 117(8), 115(8), 107(3), 91(3), 84(20), 58(65)
14	*N*-oxide-AMI	16.11	294(52)	278(5), 233(100), 218(12), 205(13), 191(22), 155(13), 117(35), 105(32), 91(29), 55(7), 45(8), 588(13), 316(10)
15	Dihydroxyl-AMI	16.24	310(69)	292(26), 231(100), 216(60), 185(27), 171(24), 115(20), 93(53), 58(35)
16	Met-16	16.66	?	344(64), 304(74), 262(34), 231(100), 216(33), 191(32), 172(11), 153(8), 105(9), 86(16), 45(29)
17[a)]	Met-17	~9.0	250(73)	233(100), 218(30), 191(43), 155(8), 117(40), 105(41), 91(36)

a) Data from another run with higher stacking effect.
See Fig. 1 of preceeding paper [1] for ring numbering scheme of amitryptyline

higher injection amounts (more stacking effects). Its spectrum indicated that it could be a demethylated nortryptyline.

4 Concluding remarks

Nonaqueous CE was coupled with ESI-MS using a commercial ion spray interface to profile tricyclic antidepressants of similar structures and mass-to-charge ratios. Pure methanol generates stable ion spray for the nonaqueous CE separation. Once optimized, the system generated detection limits of 3 pg of metabolites injected onto the capillary. Most importantly, the ESI spectra of the tricyclic antidepressants provide valuable structural information for the identification of the compounds and their metabolites produced by the fungus *Cunninghamella elegans*. Sixteen metabolites were detected and most of them were tentatively identified as demethylated and/or hydroxylated, and/or *N*-oxidized products.

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