Normal-Phase Automated Mass-Directed HPLC Purification of a Pyrrolobenzodiazepine Library with Vasopressin Agonist Activity

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A 23-member library of pyrrolobenzodiazepine derivatives with vasopressin agonist activity was purified on a 100-mg per injection scale using normal-phase (NP) automated mass-directed HPLC. Analytical NP APCI-LC/MS on an experimental monolith silica CN column utilizing gradients of methanol in ethoxynonafluorobutane (hexane-like solvent) was used to provide data on chromatographic purity and ionization of the solutes. The analytical data collected were used to program a preparative LC/MS instrument for "smart" fraction collection based on the protonated molecular ion of the component of interest. Preparative HPLC was carried out on a preparative cyano column with gradients of polar organic solvents in heptane containing n-propylamine as a basic additive. Flow rates twice as high as conventional ones were used for purification of library compounds. Small aliquots of the preparative flow were mixed with makeup solvent and introduced into an APCI source of a quadrupole mass spectrometer, which triggered collection of solutes. Two methods with fixed instrument parameters were used for purification. The system utilized commercially available instrumentation and software, which provided excellent recovery and purity of the library components and appeared to be useful as a fast and efficient alternative to traditional purification technologies based on reversed-phase LC/MS.

Introduction

Despite the tremendous progress of modern combinatorial chemistry allowing production of numerous compounds in a short period of time, their purification remains a bottleneck in medicinal chemistry. A recent review[1] summarized the efforts to provide compounds of sufficient purity at the early stages of drug discovery. A number of automated methods for purification, mostly based on the combination of reversed-phase HPLC and/or UV and mass spectrometry for detection are reported in the literature.[2−17] The popularity of such methods can be attributed to the availability of a wide variety of RP columns and their efficiency, as well as to a simplified chromatographic method development and ease of solute detection. Limited solubility of organic compounds in aqueous mobile phases, potential of generating multiple fractions,[1] presence of TFA in concentrated samples, and potential for instability for acid-labile compounds[18] are some of the drawbacks of this, by far, most popular approach to purification.

Normal-phase chromatographic techniques, such as HPLC and supercritical fluid chromatography, can provide significantly better solubility, complementary chromatographic selectivity, and faster and safe sample recovery, as well as lower pressure and higher flow rates.[19−22] While silica gel is the packing material most frequently associated with NP separations, an HPLC column made of silica cannot be used for HPLC of basic compounds because of severe tailing and the amount of mobile phase needed for column equilibration (20−40 column volumes).[23] These problems can be easily avoided by using polar bonded stationary phases, such as cyano and diol packing materials, and mobile phases with basic additives.[24]

In the past, flammability of hexane-based mobile phases in APCI and the fact that they do not support solute's ionization in ESI mode presented a problem for the use of NP HPLC in combination with modern mass spectrometry.

In the last several years, we've shown that hexane can be replaced in NP separations with ethoxynonafluorobutane (ENFB), a nonpolar, nonflammable, environmentally friendly solvent, which is completely miscible with most

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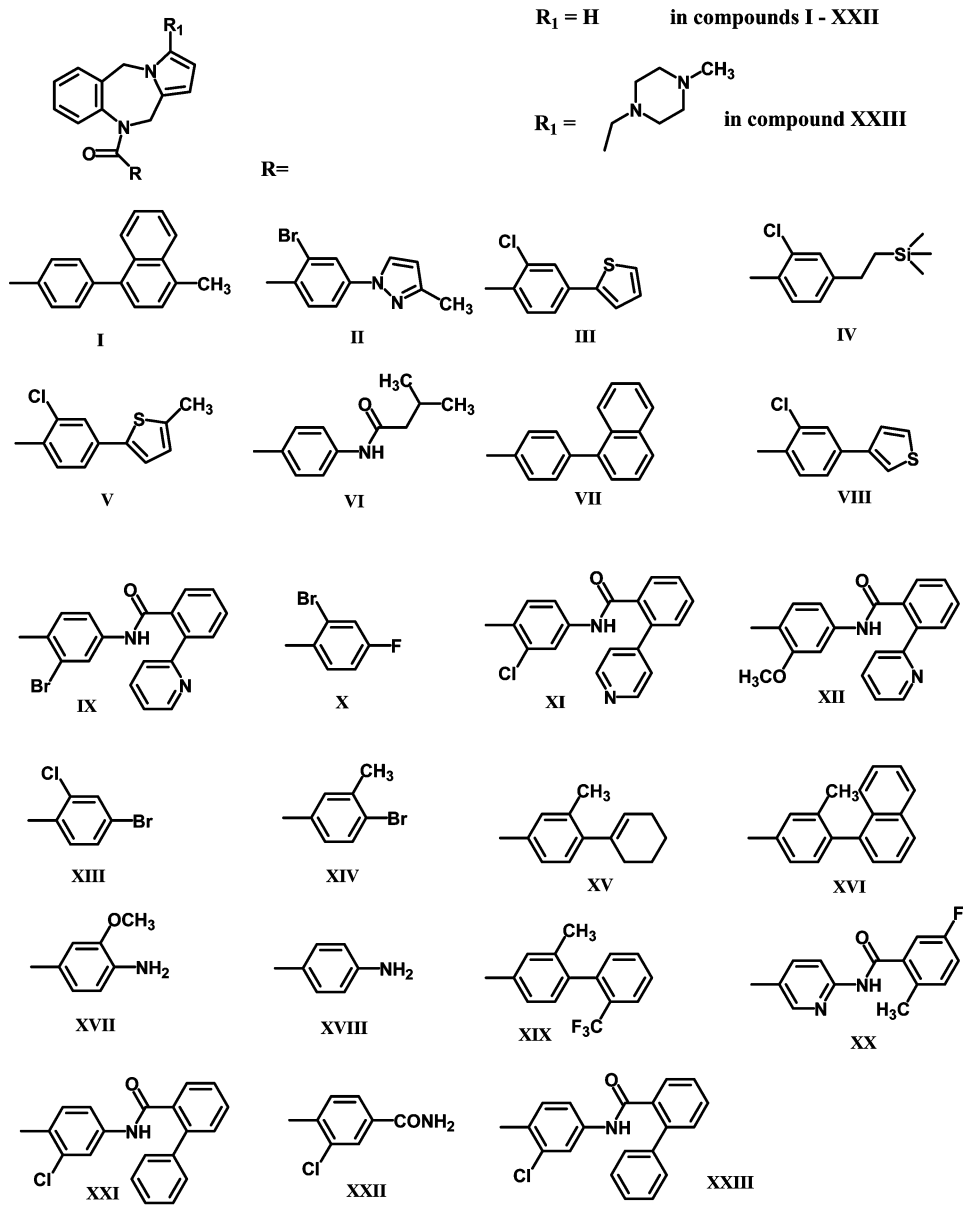

Figure 1

Figure 1. Structures of the library compounds **I**−**XXIII**.

other solvents.[25] We also described applications of mobile phases based on ENFB for analytical, preparative and chiral HPLC and LC/MS.[25−29] This solvent was recently used by others for chiral and bioanalytical work.[30,31]

NP chromatographic methods combined with mass spectrometry have not been widely used in medicinal chemistry, especially in their HPLC format. To the best of our knowledge, there is only one example in the literature where authors used NP LC/MS for analytical purposes in combinatorial library purification, with actual purification done employing UV detection.[19]

In this communication, we report mass-directed purification of a 23-member library of pyrrolobenzodiazepine derivatives (**I**−**XXIII**) (Figure 1) with vasopressin agonist activity using a NP HPLC system for separation and atmospheric pressure chemical ionization (APCI) with MS detection for fraction collection. All instrumentation used in this study was available commercially. This work was part of our broader efforts to improve purity and provide sufficient quantities (approximately 100 mg) of a significant number of structurally similar compounds for further biological evaluation. Compounds **I**−**XXIII** were prepared in the course of various Discovery research programs (Table 1), and their purity was unknown. All compounds in this group had sufficiently similar structure to warrant the expectation that they would exhibit similar chromatographic behavior. We were hoping that a minimal set of analytical and preparative methods with fixed instrumental parameters could be developed, and become the basis of a truly automated and "intelligent" chromatographic approach for purification of a particular library of synthetic compounds within a broad range of sample loads. In addition, the influence of instrument parameters on reliability of fraction collection, determination of low and

Table 1

compound number	patent issued/publication
I	WO 2002083683 A1; WO 2002083680 A1; WO 2002083678 A1
II	US 6511974 B1; WO 2001022969 A2; WO 9906409 A1
III	US 6268360 B1; WO 2000046227 A1
IV	US 6511974 B1; WO 2001022969 A2
V	US 6268360 B1; WO 2000046227 a1
VI	US 5736540 A; US 5516774 A
VII	WO 2002083683 A1; WO 2002083680 A1; WO 2002083678 A1
VIII	US 6268360 B1; WO 2000046227
IX	US 5880122 A; WO 9820011 A1; WO 9749707 A1; US 5700796 A
X	US 6511974 B1; WO 2001022969 A2; WO 9906409 A1
XI	US 5880122 A; WO 9749707 A1; US 5700796 A
XII	US 5880122 A; WO 9820011 A1; WO 9749707 A1; US 5700796 A US 6511974 B1; WO 2002083683 A1; WO 2002083681 A1;
XIII	WO 2002083680 A1; WO 2002083678 A1; US 6268360 B1; WO 2001022969 A2; WO 2000046227 A1; WO 9906409 A1; WO 2002083684 A1; WO 2002083683 A1; WO 2002083682 A1;
XIV	WO 2002083681 A1; WO 2002083680 A1; WO 2002083679 A1; WO 2002083678 A1; WO 2002083684 A1; WO 2002083682 A1; WO 2002083681 A1;
XV	WO 2002083679 A1
XVI	WO 2002083682 A1; WO 2002083681 A1; WO 2002083679 A1
XVII	WO2002083678 A1; US 5736540 A; J. Med. Chem., 1988, 41(14), 2442−2444; US 5733905 A; US 5516774 A; EP 636625 A2 J. Med. Chem. 1998, 41(14), 2442−2444;
XVIII	US 5753648 A; US 5736540 A; US 5733905 A; US 5700796 A; US 5516774 A; EP 636625 A2
XIX	WO 2002083683 A1; WO 2002083680 A1; WO 2002083678 A1 Bioorg. Med. Chem. Lett. 1999, 9(13), 1737−1740;
XX	US 5753648 A; WO 9749707 A1; US 5700796 A; US 5536718 A Bioorg. Med. Chem. Lett. 2000, 10(8), 695−698;
XXI	US 5880122 A; US 5753648 A; WO 9820011 A1; WO 9749707 A1; US 5700796 A; US 5536718 A
XXII	US 6511974 B1; WO 2001022969 A2; WO 9906409 A1
XXIII	Bioorg. Med. Chem. Lett. 2000 10(8), 783−786; US 5753648 A; WO 9749707 A1; US 5700796 A

high limits of loadability and productivity, and recovery of such a purification system were investigated in detail.

Experimental Section

Solvents and Chemicals. HPLC grade heptane, methylene chloride (CH_2Cl_2), methanol (MeOH), acetonitrile (ACN), and ethanol were purchased from EM Science (Gibbstown, NJ). *n*-Propylamine (NPA), dimethylsulfoxide (DMSO), acetic acid, and 2 M ammonia in methanol were purchased from Sigma-Aldrich (St. Louis, MO).

Ethoxynonafluorobutane (ENFB) was obtained as 3 M Novec Engineered Fluid HFE-7200 from 3 M Company (St. Paul, MN).[25]

Compounds **I**−**XXIII** (Figure 1) were prepared using published procedures (Table 1).

Analytical NP LC/MS Instrumentation. An 1100 Series LC/MSD (VL model, Agilent Technologies, CA) detector equipped with APCI source and combined with Agilent's 1100 Series autosampler (model G1329A), thermostatted column compartment (G1316A), diode-array detector (G1315A), and quaternary pump (model G1311A) was used for analytical normal-phase LC/MS. The instrument was operated using Agilent's ChemStation (version B.02.01-SR1) and Easy Access (G2725AA, version A.04.02) software.

An experimental monolith silica CN column (0.46 × 10 cm) (courtesy of Merck KGaA, Darmstadt, Germany) was used for analytical LC/MS experiments. Linear 4-min gradients of MeOH (Table 2) (containing ∼0.1% ammonia) in ENFB were run at 4 mL/min flow rate (10 column volumes) followed by a wash step with 100% MeOH/ammonia for 2 min (∼5 column volumes). Before each run the column was equilibrated with ∼5 column volumes of the starting solvent. Samples were dissolved in CH_2Cl_2 to prepare solutions with concentration ∼1 mg/mL, with injection volume being 5 μL.

The eluent from the monolith silica column was split 3:1 using a back-pressure regulator (Upchurch, 100 psi) positioned between the diode-array detector and the APCI source, delivering 1 mL/min to the source.

The APCI source was kept at the following conditions: drying gas flow rate 4 L/min; nebulizer gas pressure 60 psi; drying gas temperature 300 °C; vaporizer temperature 350 °C; capillary voltage 4,000 V, corona current 4 μA. Mass spectra were measured in a positive scan mode (200−1,000 *m*/*z*) with the fragmentor set at 70, gain at 1, threshold at 150, step size at 0.3, peak width at 0.05 min, and cycle time at 0.48 s/cycle. Gradients used: analytical A, 5−20%; B, 10−30%; C, 10−50%; preparative D, 5−20%; E, 10−50%.

Auto-Preparative LC/MS Setup. Preparative LC/MS was carried out on an Agilent 1100 preparative LC/MS system composed of a LC/MSD (VL model) equipped with APCI source, two isocratic pumps (G1361A), autosampler (G2260A), diode array detector (G1315B, flow path 0.3 mm), fraction collector (G1364A) equipped with funnel tray (G1364−84532), active splitter (G1968C, version 1.0.7), and an additional isocratic pump (G1310A) connected to the analytical side of the active splitter. ChemStation software (version A.10.02) was used for instrument control, data acquisition and fraction collection.

The APCI source spray chamber parameters were the same as for the analytical LC/MS system. Mass spectra were measured in a positive scan mode (200−800 *m*/*z*) with the fragmentor set at 80, gain at 1, threshold at 250, step size at 0.1, peak width at 0.25 min, cycle time at 2.34 s/cycle with "Time Filter" feature enabled. The fraction collection parameter in the MS Detector menu was set to maximum peak duration of 1.25 min and with the "Peak-based" and "MSD Used for Mass-Based Collection" features enabled. Fraction collection in a fraction collector menu was set up with "Use Sample Target Masses" and "Detectors: MS only" features enabled and parameters set as follows: minimum

Table 2

compound no.	MW	most intense ion in MS	purity at 254 nm %	analytical gradient	analytical RT min	preparative gradient	preparative RT min	collected[a] mg	injection solvent	peak volume mL
I	428	429	92	A	1.6	D	5.8	95	solvent B[b]	45
II	446	447	96	A	2.1	D	6.4	97	CH_2Cl_2	39
III	404	405	96	A	1.9	D	5.7	95	CH_2Cl_2	50
IV	418	419	65	A	1.0	D	3.7	58	CH_2Cl_2	29
V	418	419	98	A	1.6	D	6.7	95	solvent B	45
VI	387	388	98	A	2.6	D	16.3	96	solvent B	50
VII	414	415	98	A	1.7	D	5.7	95	solvent B	45
VIII	404	405	59	A	2.2	D	8.2	63	solvent B	39
IX	564	565	99	A	4.0	E	9.7	95	DMSO	50
X	384	385	29	A	1.3	D	6.7	26	solvent B	19
XI	518	519	83	B	3.0	E	12.7	87	solvent B	36
XII	515	516	98	A	3.8	E	5.7	92	solvent B	45
XIII	402	403	66	A	1.5	D	6.4	68	CH_2Cl_2	30
XIV	478	479	99	A	2.1	D	8.0	76	CH_2Cl_2	50
XV	382	383	94	A	1.0	D	5.1	79	solvent B	50
XVI	430	431	90	A	1.3	D	5.7	97	solvent B	50
XVII	333	334	99	A	2.5	D	13.1	96	solvent B	50
XVIII	303	304	57	A	3.0	E	9.8	37	solvent B	50
XIX	446	447	97	A	1.1	D	6.4	97	CH_2Cl_2	50
XX	440	441	96	A	2.9	E	7.7	93	DMSO	50
XXI	518	519	99	A	3.8	E	9.8	72	DMSO	31
XXII	365	366	98	A	3.6	E	12.5	96	DMSO	47
XXIII	630	530	69	C	2.3	E	9.9	67	DMSO	39

[a] collected from a 100-mg sample injected in ~0.6 mL of the injection solvent. [b] CH_2Cl_2-MeOH 8:2 (0.1% NPA) (Experimental).

and maximum peak widths 0.09 and 1.25 min, respectively; peak slope 0, threshold 20 000, collector delay 0.1 min, mass window 2 units.

Purification of the samples was carried out on a Luna CN 2 × 15 cm preparative HPLC column packed with 5 μm particles (Phenomenex, Torrance, CA). Two methods employing linear 11-min gradients at 40 mL/min flow rate of solvent A (heptane/CH_2Cl_2, 9:1, containing 0.1% NPA) and solvent B (CH_2Cl_2/MeOH, 8:2, 0.1% NPA) were used for purification: 5−20% and 10−50% of solvent B in solvent A. The void volume of the column (~42 mL) was measured experimentally by injecting nonretained toluene followed by elution with 10% of solvent B in solvent A.

Samples of compounds **I**−**XXIII** (100 mg) were dissolved in 0.5 mL of either CH_2Cl_2 or solvent B/DMSO (see Table 2) and injected from Agilent's high-recovery injection vials (part No. 5183−2030) with injection volume set at 850 μL. Before each injection the preparative column was equilibrated with ~4 column volumes of the starting solvent (3 min at 40 mL/min and ~1 min of the injection routine). After the end of the gradient was reached, the column was washed with 100% solvent B at 40 mL/min for 3 min.

Column eluent (flow rate 40 mL/min) was split using an active splitter (ratio 1:10 606), with the analytical part of the flow being mixed with a makeup solution (mixture of ethanol and MeOH, 7:3 with 0.1% of acetic acid added, 1 mL/min) and directed to the APCI source. Preparative flow was directed to the fraction collector via the preparative side of the active splitter using an additional Teflon capillary tubing (volume ~3.2 mL). This was done to compensate for the ~5 s delay time between when a solute left the column and the moment when its maximum concentration was detected by the APCI-MS hardware.

Purified fractions of the library constituents were concentrated and dried in vacuum to determine their weight and purity.

Analytical RP LC/MS Instrumentation. The purity of the compounds obtained after preparative LC/MS was assessed on an open access LC/MS system consisting of Agilent's 1200 Series MSD detector (G2735, SL version) equipped with an ESI source and combined with a degasser (G1379B), an autosampler (G1329B), a thermostatted column compartment (G1316A), a diode-array detector (G1315B), and a binary pump (G1312B). The instrument was operated using ChemStation (B.02.01) and Easy Access (A.04.02) software. Two Chromolith RP18 columns (2 × 50 mm) (Merck KGaA) connected in series were used for analysis with methods employing a 3-min gradient of 10−100% of ACN in water (both containing 0.05% formic acid) at 1 mL/min flow rate. The chromatographic purity was evaluated at 254 nm using the instrument's software.

Discussion

Compounds **I**−**XXIII** could be considered as typical drug-like molecules.[32] Some of the library members (**IV**, **X**, **XIII**−**XVI**) are hydrophobic (as free bases), whereas others (**VI**, **IX**, **XII**, **XX**−**XXIII**) are more polar. Most of the compounds were soluble in CH_2Cl_2 or mixtures of MeOH and CH_2Cl_2, except for **IX** and **XX**−**XXIII**, which could form concentrated solutions only in DMSO (Table 2).

We reported recently that gradients of polar organic solvents in hydrocarbons (hexane, heptane) or hexane-like ENFB on polar bonded phases (cyano, diol) were successfully used for analytical and preparative HPLC and LC/MS of a wide variety of organic compounds.[25−29] Such an approach was used to purify compounds **I**−**XXIII**.

Purity Assessment of Compounds I−**XXIII and Their Ability to Produce Ions by Analytical NP APCI-LCMS.** Evaluation of purity (chromatographic homogeneity at 254 nm), retention and measurement of positive APCI spectra of the library compounds **I**−**XXIII** was carried out on a cyano column in a manner similar to the procedure

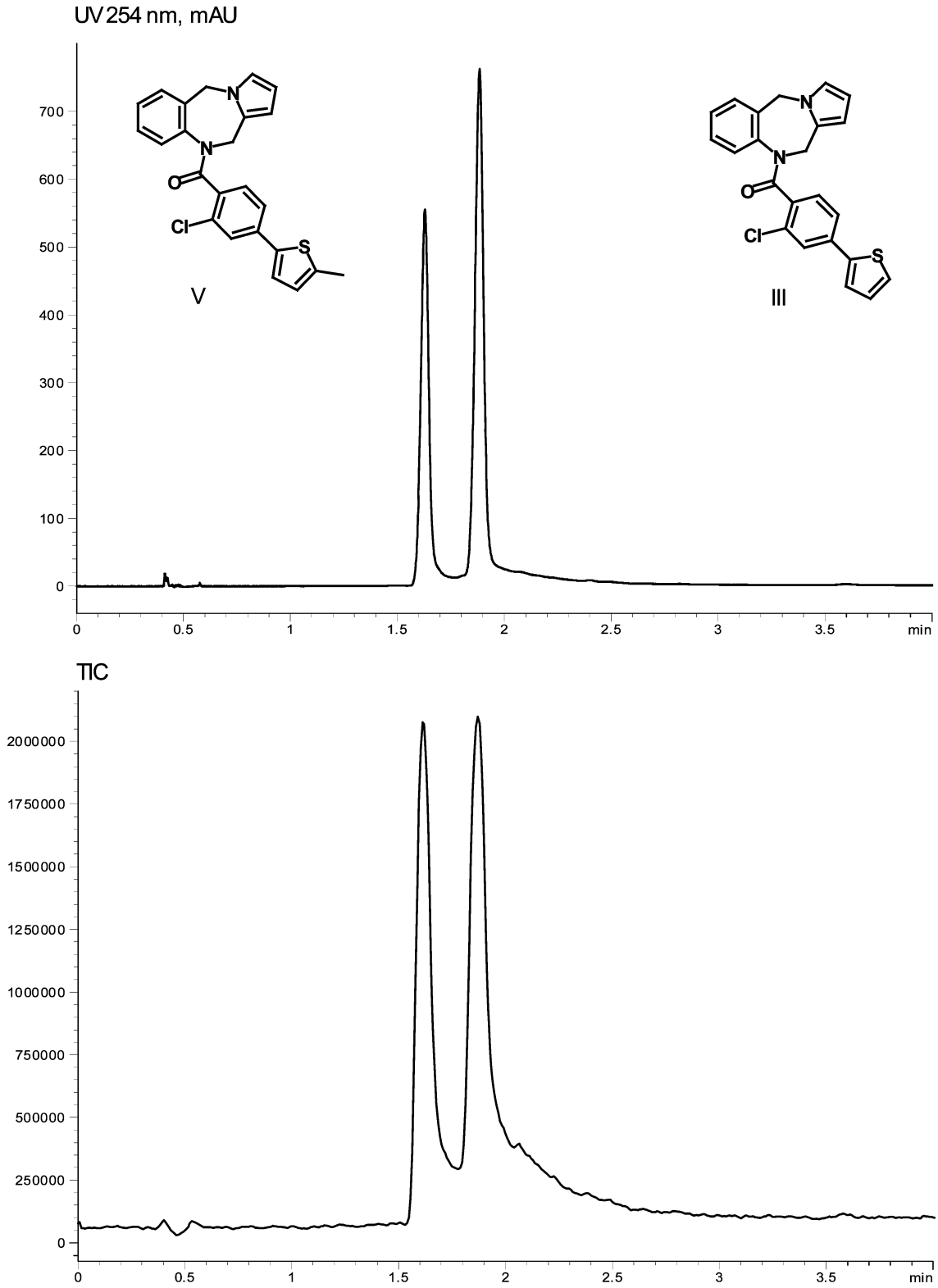

Figure 2. NP analytical LC/MS of compounds **III** and **V** on a monolith silica CN column using 5−20% gradient (see Experimental Section for chromatographic and MS conditions).

described earlier;[27] the results are summarized in Table 2. We used an experimental 4.6 × 100 mm monolith silica CN column, kindly provided by Merck KGaA, Darmstadt, Germany. Recently commercialized Chromolith C18 and silica gel columns[33] have been reported to exhibit high chromatographic performance at elevated flow rates. We found the experimental monolith silica CN column to be an excellent tool for quick assessment of the purity of our library compounds in NP mode. For example, it provided good selectivity and resolution between **III** and its methyl analog **V** (UV and TIC traces are presented in Figure 2, and mass spectra are presented in Figure 3). Positional isomers **III** and **VIII** were also well resolved under those conditions (Table 2). It is likely that a

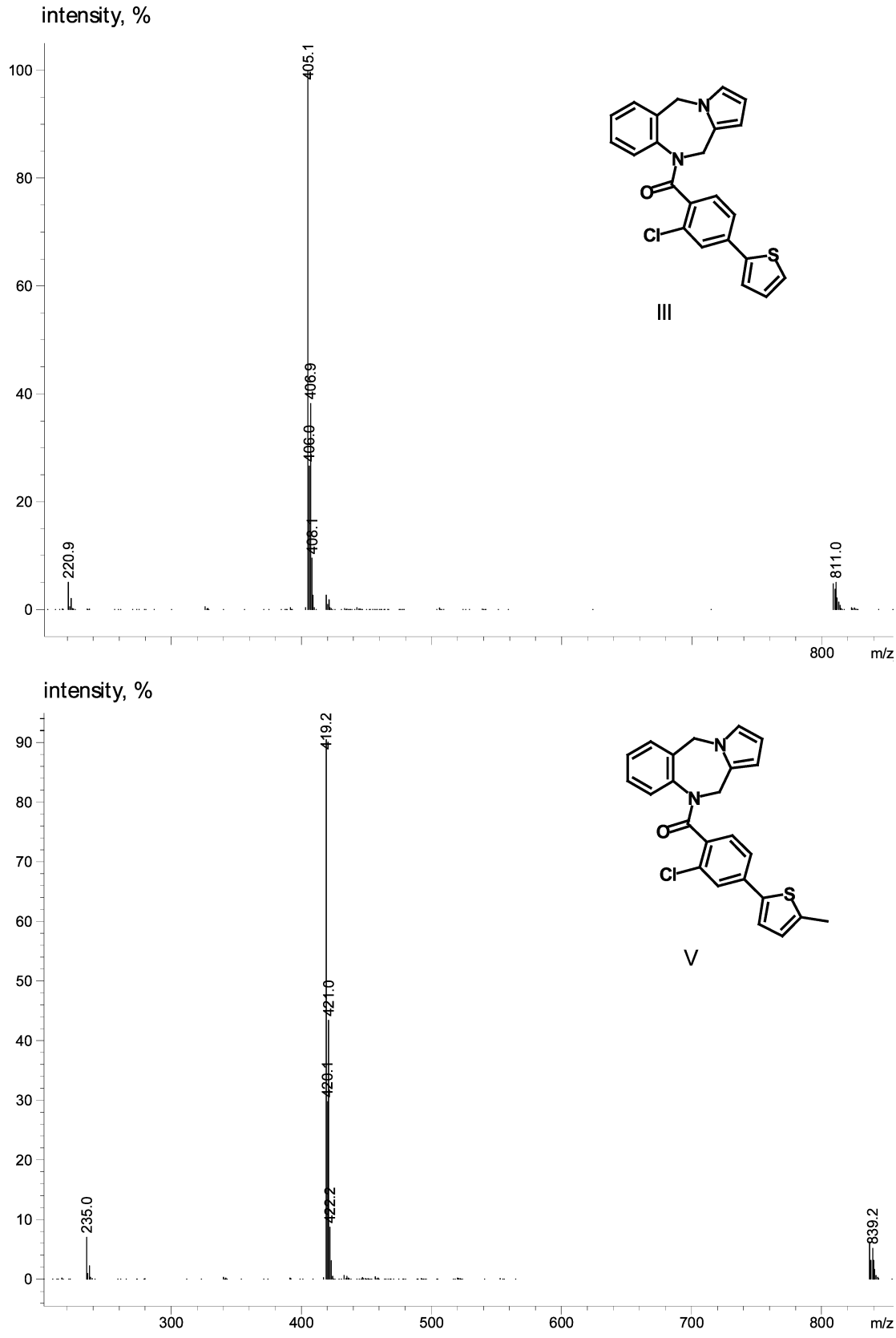

Figure 3. NP analytical LC/MS: mass spectra of compounds **III** and **V**.

conventional cyano column packed with 3−5 μm particles (e.g., Luna CN in ref 27) would be adequate for this task, at the expense of a slightly increased analysis time.

Positive APCI spectra of the compounds **I**−**XXIII** were dominated by protonated molecular ions with minimal or no fragmentation (as in ref 27; see Table 2 and Figure 3 for typical mass spectra), with one exception: the most intense ion in the spectrum of **XXIII** was an ion resulting from the loss of the *N*-methylpiperazine moiety. This information was used to program our fraction collection in preparative work.

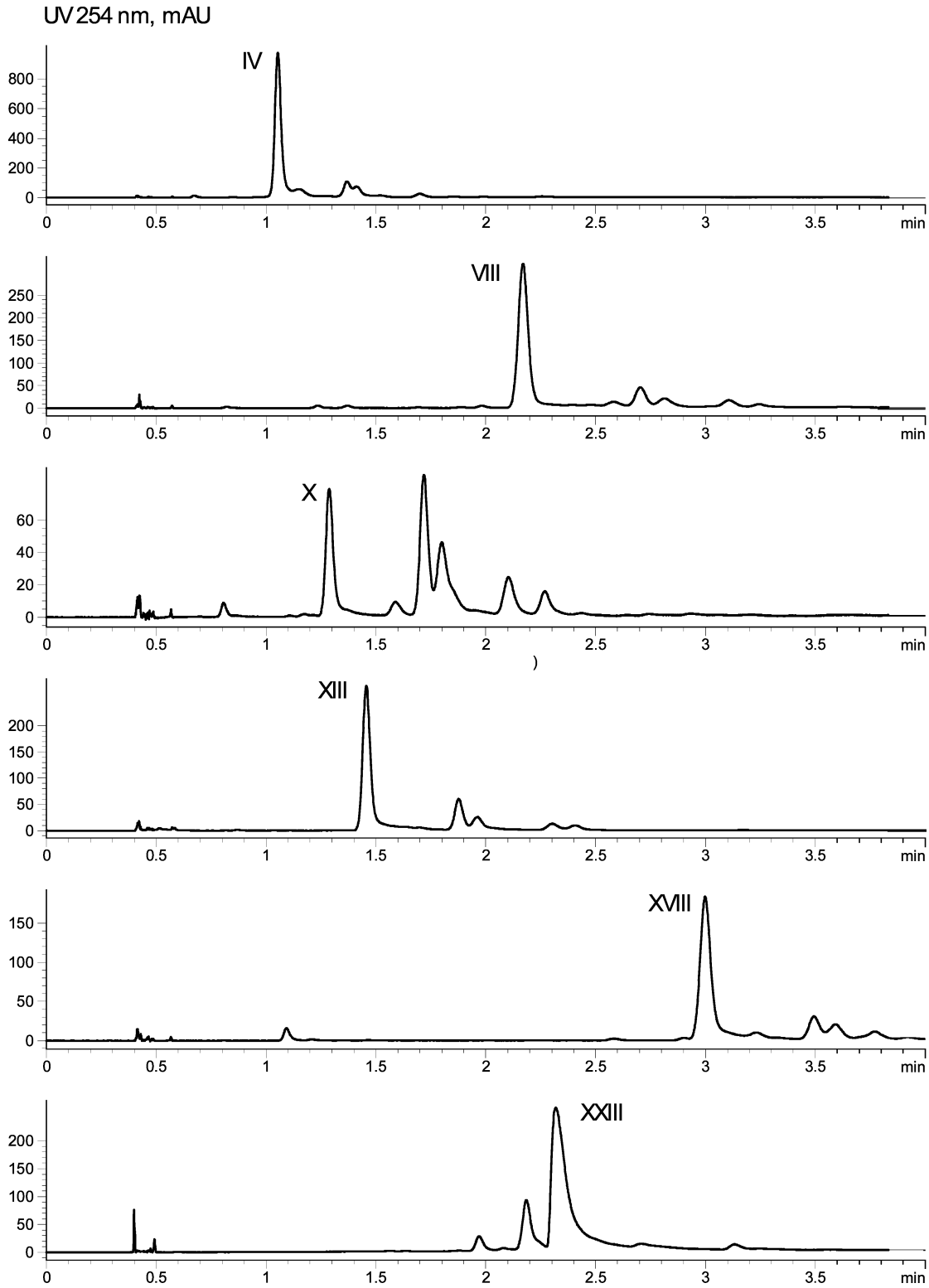

Figure 4. Purity of compounds **IV**, **VIII**, **X**, **XIII**, **XVIII**, and **XXIII** as determined by analytical LC/MS, conditions as in Figure 2, except for **XIII** (gradient 10−50%).

Initially, the 5−20% gradient was run for every library sample, while more polar gradients were used to provide adequate retention for compounds **XI** and **XXIII** (Table 2). We found that six compounds (**IV**, **VIII**, **X**, **XIII**, **XVIII**, and **XXIII**) contained substantial amounts of impurities (Figure 4) when analyzed at that concentration (∼1 mg/mL), while the level of impurities for the rest of the library was fairly minimal (purity assessment results are shown in Table 2). Overall, the analytical procedure employed was quick (∼3.5 h for the whole library) and efficient, and it provided enough information on retention, purity and APCI spectra for all library components.

Mass-Directed Purification of Compounds I−XXIII. Mobile Phases and Preparative HPLC Column. Recently, we applied NP chromatographic methods to preparative separations of pharmaceutical intermediates on cyano col-

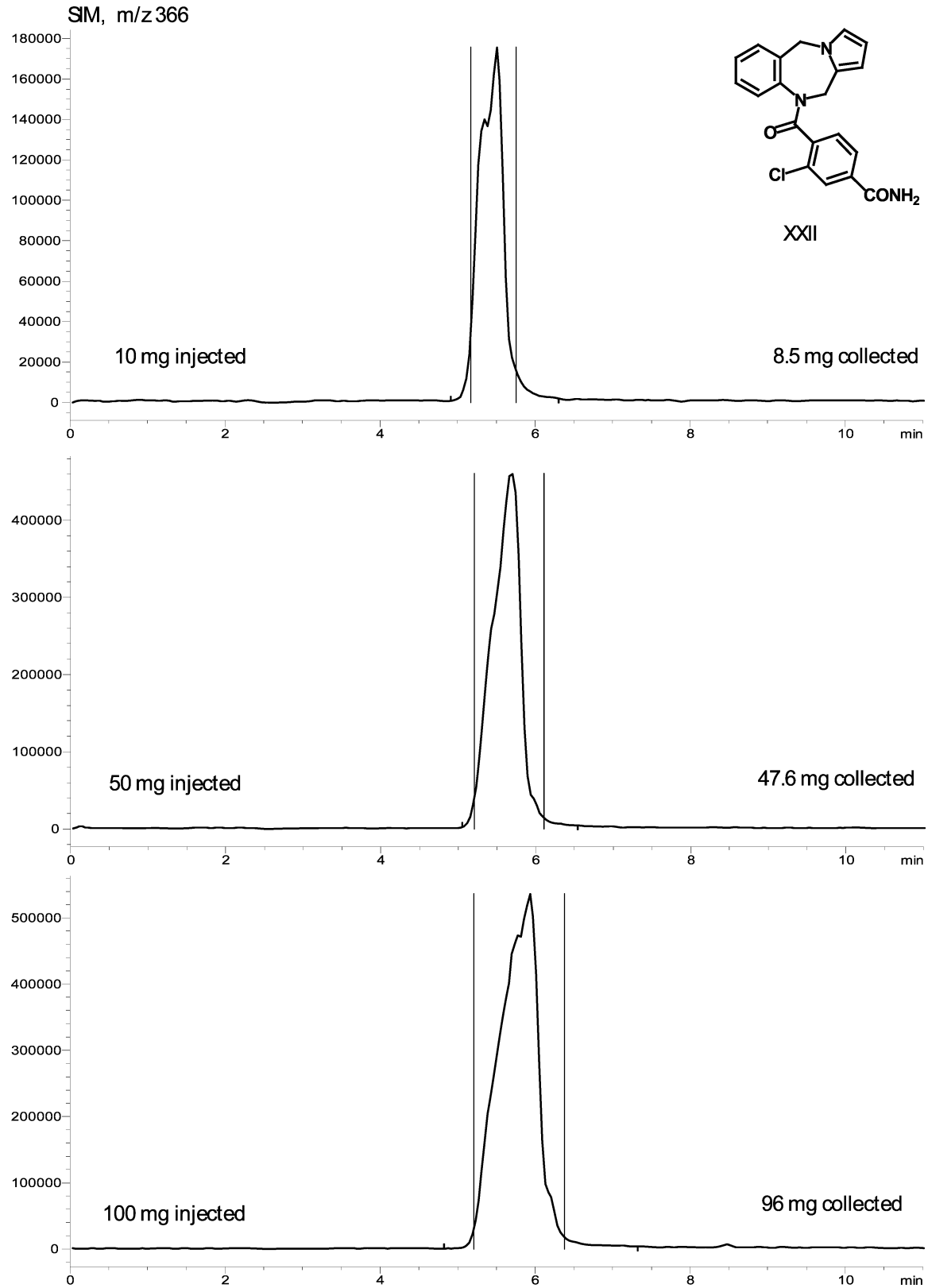

Figure 5. Purification system calibration with 10, 50, and 100 mg of compound **XXII** injected in 0.5 mL of DMSO. Preparative gradient 10−50%, fraction collection threshold: 20 000; for other conditions see Experimental Section.

umns[29] and to mass-directed preparative HPLC of steroids and biologically active acidic and basic substances and drugs,[26] as well as to chiral NP separation of non-UV-active pharmaceutically relevant racemic mixtures.[28] Preparative gradients of polar solvents in heptane on preparative cyano column with 0.1% of a basic additive, NPA (see ref 24), were chosen as a general strategy for mass-directed purification of compounds **I**−**XXIII**.

Data on chromatographic retention and APCI ionization of the library compounds acquired in the course of analytical

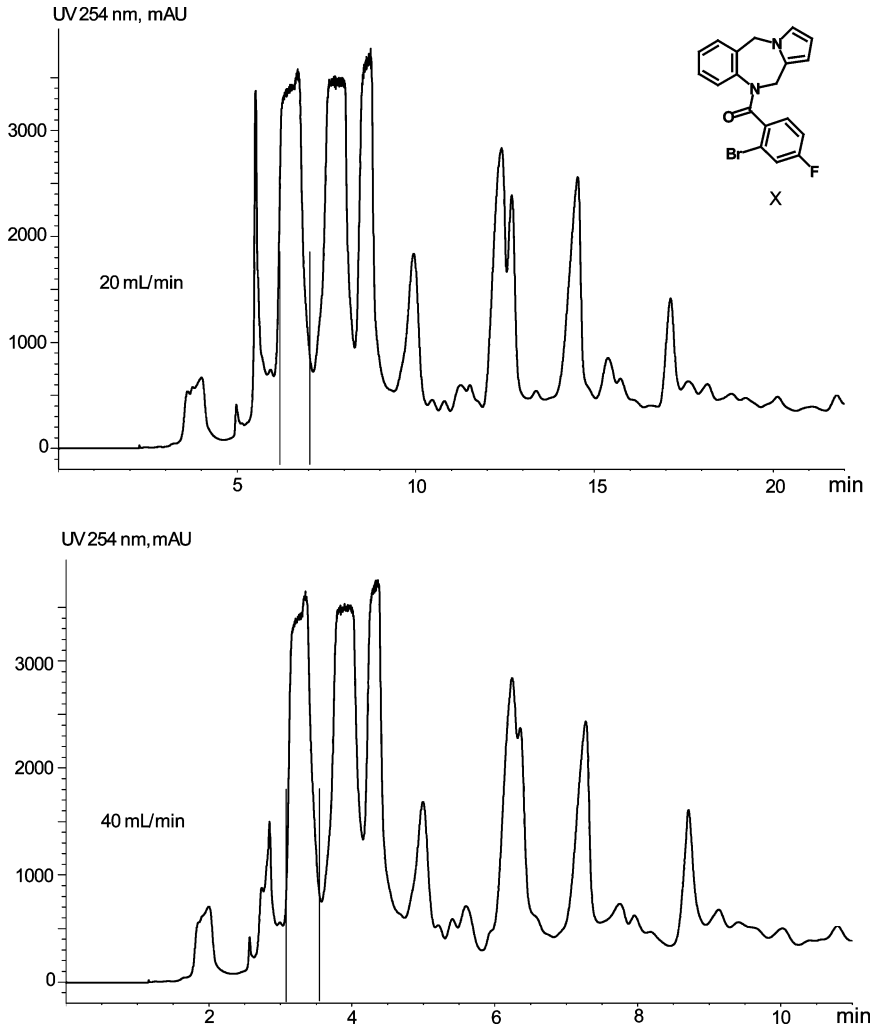

Figure 6. NP HPLC of 100 mg of compound **X** at 20 and 40 mL/min on a preparative Luna CN column, vertical lines indicate time when peak collection was triggered (upper trace: 29 mg collected, lower trace: 26.5 mg).

NP LC/MS experiments was used to select appropriate gradient conditions for elution, and the predominant ion in the mass spectrum was used to program fraction collection of the components of interest.

Selection of solvents A and B (see Experimental Section) was based on the assumption that chromatographic mechanisms involved remained essentially the same when experimental monolith silica CN column (used for analytical work) was replaced with the Luna CN preparative column. The same argument could be made for switching to gradients of mixtures of CH_2Cl_2 with MeOH in heptane for preparative purposes from analytical gradients of MeOH in ENFB, with the relatively high cost of ENFB being a significant factor to consider.

Initial preparative HPLC runs with compounds weakly retained on the analytical column (for example, **IV**) showed that they exhibited similar low retention on the preparative Luna CN when 5−20% gradient of solvent B in solvent A was employed (Table 2). Therefore, this method was applied to purification of **I**−**VIII**, **X**, **XIII**−**XVII**, and **XIX**. A 10−50% gradient of solvent B in solvent A was needed to ensure the adequate elution of more polar compounds **IX**, **XI**, **XII**, **XVIII**, and **XX**−**XXIII** from the preparative column.

The presence of NPA in the mobile phase was essential for the overall purification success because many members of the library contained one or more basic groups. We reported recently that NP HPLC of basic compounds on polar bonded phases could be easily improved with basic additives (such as NPA) in mobile phases.[24] NPA's presence in the mobile phase, in theory, may cause quenching of solute signal in the APCI source,[27] but in our preparative setup, this effect was negligible, as only a very small portion of the preparative flow was delivered to the ionization source with flow of acidified makeup solvent. Mass spectra of almost all library compounds (except for **IX**, **XI**, and **XII**, which have a pyridine group) obtained during preparative runs, did contain ions of adducts composed of the protonated molecular ion with NPA ($[MH + 59\ amu]^+$), with their intensity being ~20% of the major ion in the spectrum. The presence of such adducts had no effect on the ability of the instrument to properly detect the beginning and end of the collected fraction, which was based on the most intense ion in the spectrum. No such phenomenon was noticed in our analytical LC/MS experiments where no adducts of molecular ions with ammonia (much weaker base than NPA) have been found (see ref 27 and Experimental Section).

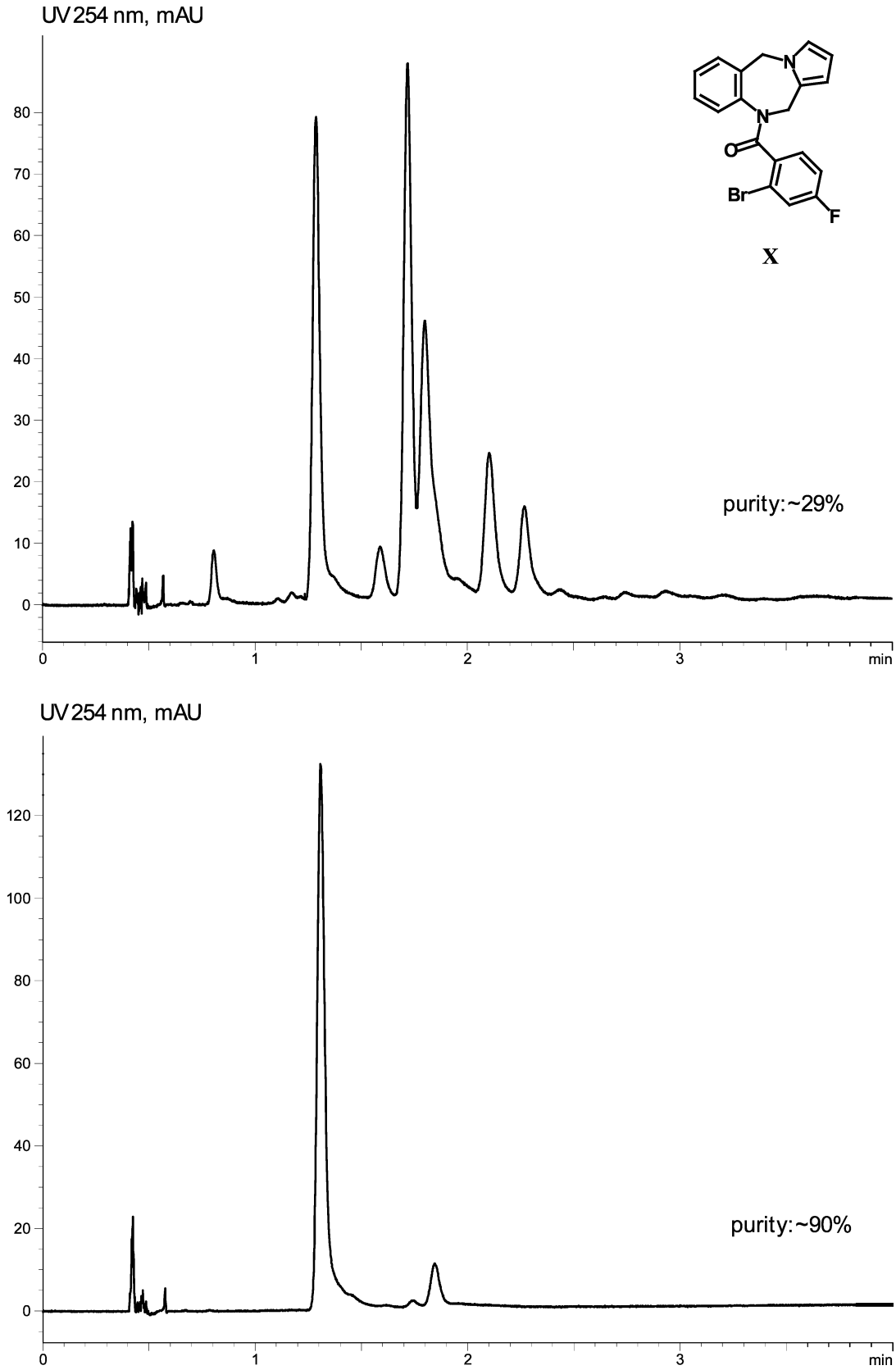

Figure 7. NP analytical LC/MS of compound **X** before and after purification (upper and lower traces, respectively), conditions as in Figure 2.

Injection Solvent and Volume. It is generally considered that the chemical nature of the injection solvent and its volume play an important role in the outcome of preparative HPLC purification. We've shown recently that up to 300 mg of a mixture of pharmaceutically relevant regioisomers could be separated in a single run on a Luna CN 2 × 15 cm column using gradients of MeOH in ENFB.[29] In that study, the injection volume was found to be a more important factor than the polarity or viscosity of the injection solvent. CH_2Cl_2, MeOH and DMSO and their mixtures were all acceptable,

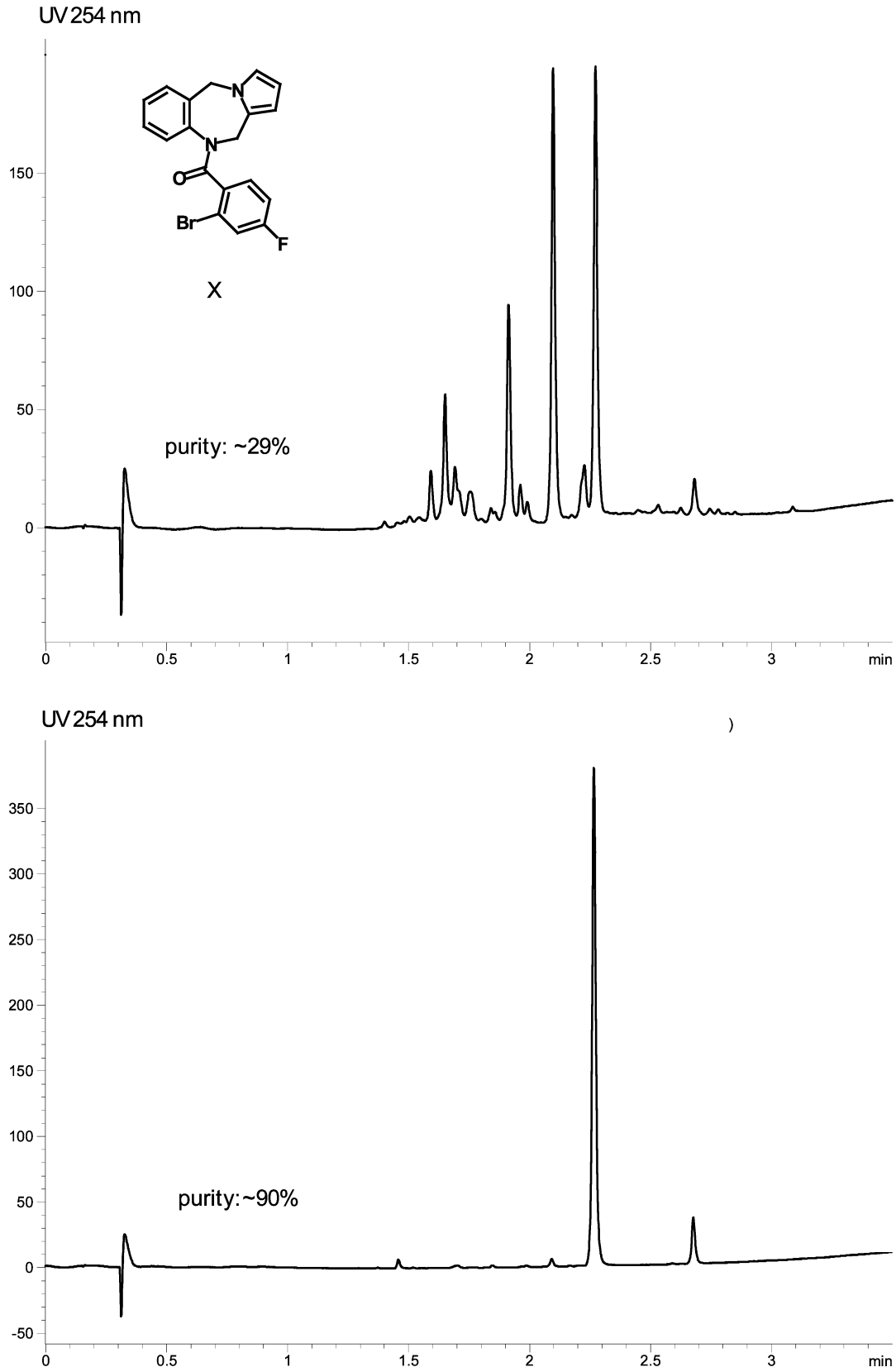

Figure 8. RP analytical LC/MS of compound **X** before and after purification (upper and lower traces, respectively), (see Experimental Section for conditions).

as long as the sample volume remained ~0.5 mL for a column of that size and a ~100-mg sample load. While setting up purification of the current library, we used CH_2Cl_2 or solvent B as injection solvents and kept the injection volume around 0.6 mL (mixture of 100 mg of the sample and 0.5 mL of the liquid) (see Table 2) for the majority of samples. Compounds **IX** and **XX**−**XXIII** were soluble only in DMSO and required the 10−50% gradient for their purification. The retention time of the DMSO peak under these conditions was ~5 min, which prevented solutes from

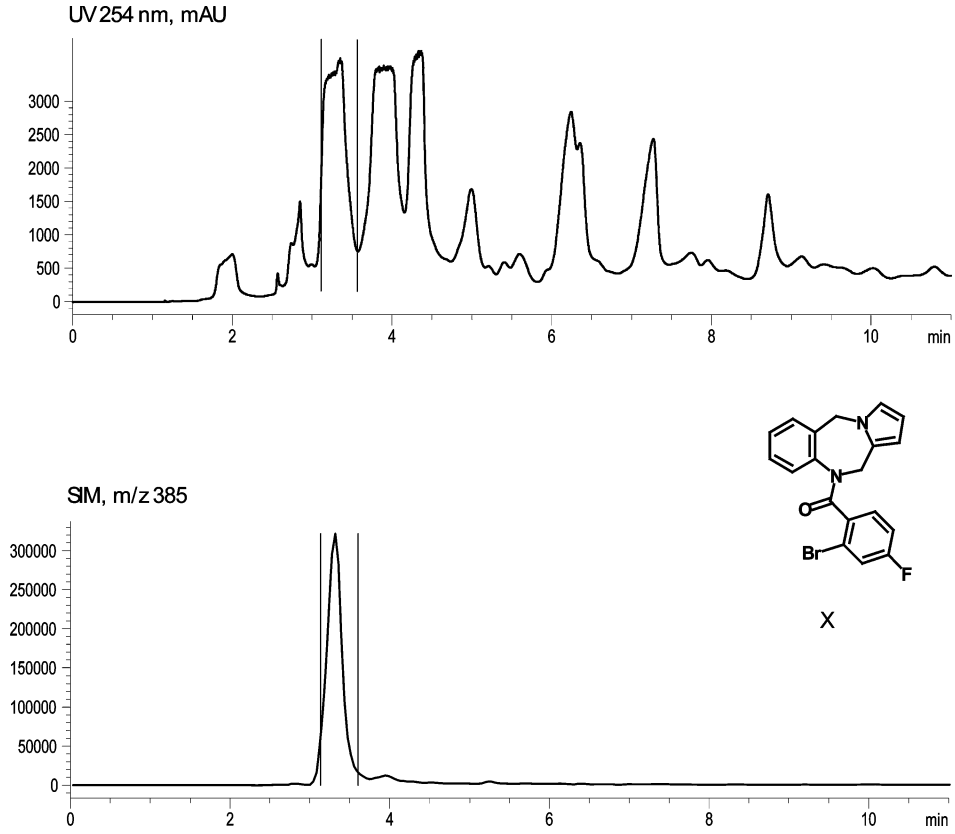

Figure 9. Automated purification of a 100-mg sample of compound **X** on a preparative Luna CN column, gradient 5−20% of solvent B in solvent A (Experimental Section), collection based on *m*/*z* 385 ion.

being contaminated with traces of DMSO because their retention time was significantly higher. In general, DMSO, frequently called a "universal" solvent, seems to be quite compatible with the purification system we describe here.

Detection and Fraction Collection. To develop a truly automated and "intelligent" purification system for a reasonably large set of similar compounds (such as combinatorial or parallel synthesis libraries) one needs to select an appropriate detection technique and intensity of the instrument's response to trigger fraction collection.

Collection of solutes based on their UV adsorption is popular and reliable but tends to create numerous fractions,[9] whereas MS-based fraction collection appears to increase fraction volume.[33] One example of a combined approach (use of UV and MS signal) is described in the literature.[34]

In the preliminary experiments, we investigated the use of the "level only" principle of a MS-driven fraction collection. In this technique, a mass spectrometer monitors the signal level from the most intense ion in the APCI-produced mass spectrum and turns the fraction collection "on" and "off" when the level of the signal exceeds or drops below a certain threshold. The purification system was calibrated by injecting 10-, 50- and 100-mg samples of almost pure compound **XXII** and measuring the level of its protonated molecular ion signal (single ion monitoring, SIM) at *m*/*z* 366 (Figure 5). A threshold of 20 000 units was found to be adequate to detect a 10-mg sample and trigger fraction collection. This threshold was used in purification of all compounds **I**−**XXIII**.

We found that stability of both total ion current (TIC) and single ion of interest (SIM) signals were both greatly affected by the instrument settings, especially when high concentrations of the solute were being purified. It was necessary to keep scan time relatively long (2.34 s) and the "Time Filter" feature enabled to produce smooth and stable TIC and SIM signals for all compounds in the library. The sensitivity of the system was substantially lower (as compared to shorter scan times and disabled "Time Filter" feature) but was still adequate to detect and collect 10-mg quantities of the solutes. Overall, this set of instrument parameters (described in the Experimental Section) was used throughout purification of the whole library.

Recovery of the Solutes. An "ideal" automated MS-driven purification system should be designed and optimized in a way to provide not only the required purity for sample components but also to minimize the inevitable loss of material from separated chromatographic peaks caused by factors such as incomplete injection and nonideal peak detection algorithm and fraction collection. We found that use of high-recovery injection vials (with conical bottoms) allowed for injection of ∼99% of the sample mass, even in the case of rather viscous samples, such as solutions in DMSO. Programmed injection volume (0.85 mL) exceeded slightly the actual volume of the sample (0.6−0.7 mL) to facilitate almost quantitative transfer of the sample onto the purification column. Presence of a small air bubble in the actual injection plug did not affect solute peak shape.

Special attention was paid to the connection between the active splitter and fraction collector to compensate for the delay between the signal from mass spectrometer and its proper collection time (see Experimental Section and

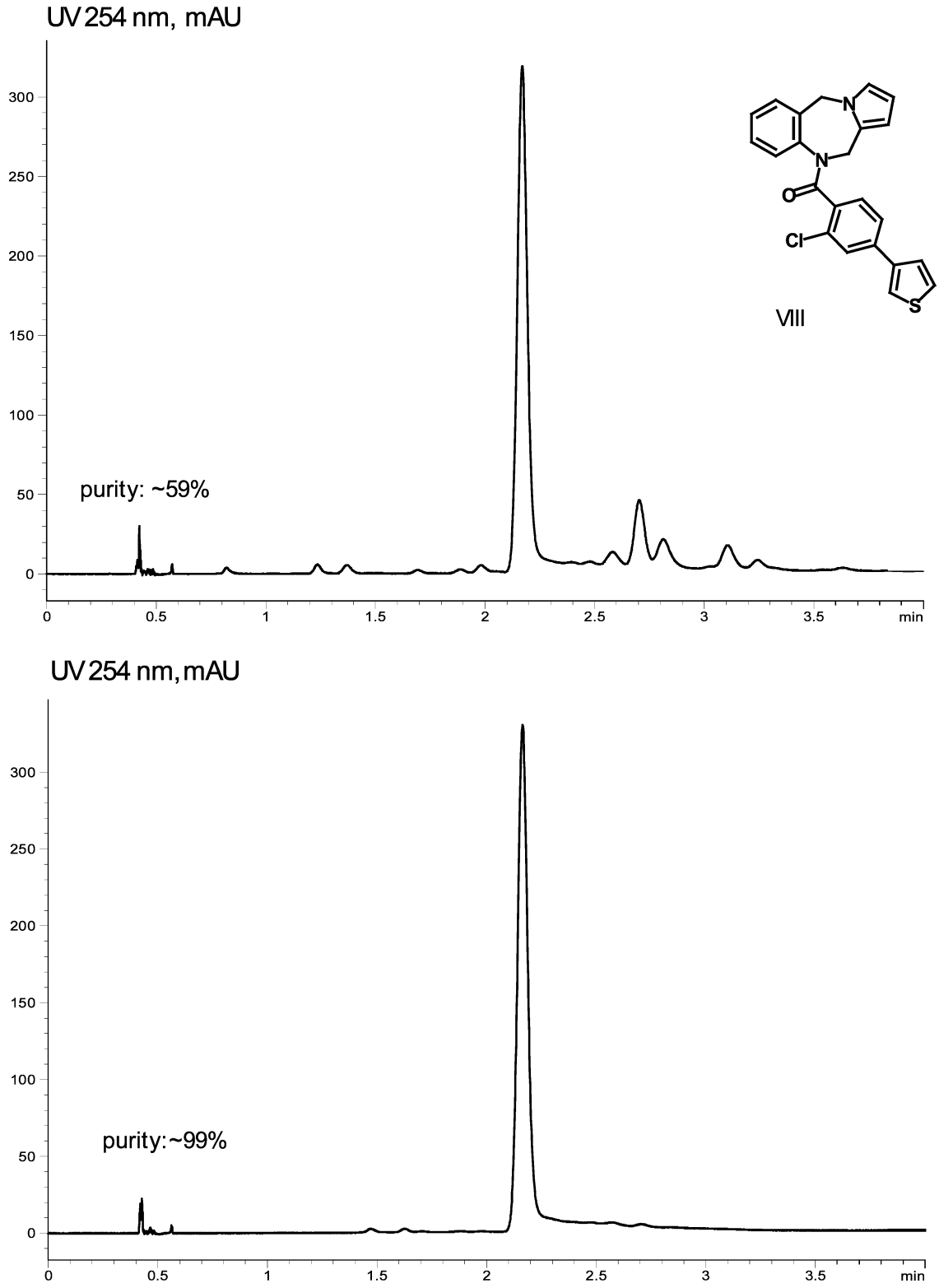

Figure 10. NP analytical LC/MS of compound **VIII** before and after purification (upper and lower traces, respectively), conditions as in Figure 2.

Discussion in ref 10). Ultimately, the system was tested (Figure 5) by injecting increasing amounts of sufficiently homogeneous compound **XXII** (10, 50, and 100 mg), while keeping all instrument parameters constant. Collected fractions were concentrated in vacuo and produced 8.5, 47.6, and 96 mg (respectively) of pure **XXII**, indicating excellent recovery overall (~85−95%), even at the lower end of the sample load range. These observations established the operational limits (with regard to sample weight) for our purification system.

We addressed the problem of improper peak detection by selecting a SIM signal threshold set at 20 000 units (with scan cycle time 2.34 s and "Time Filter" parameter enabled in the ChemStation fraction collection software menu). This setting would trigger the collection of a peak of interest when a 10-mg sample of almost pure **XXII**

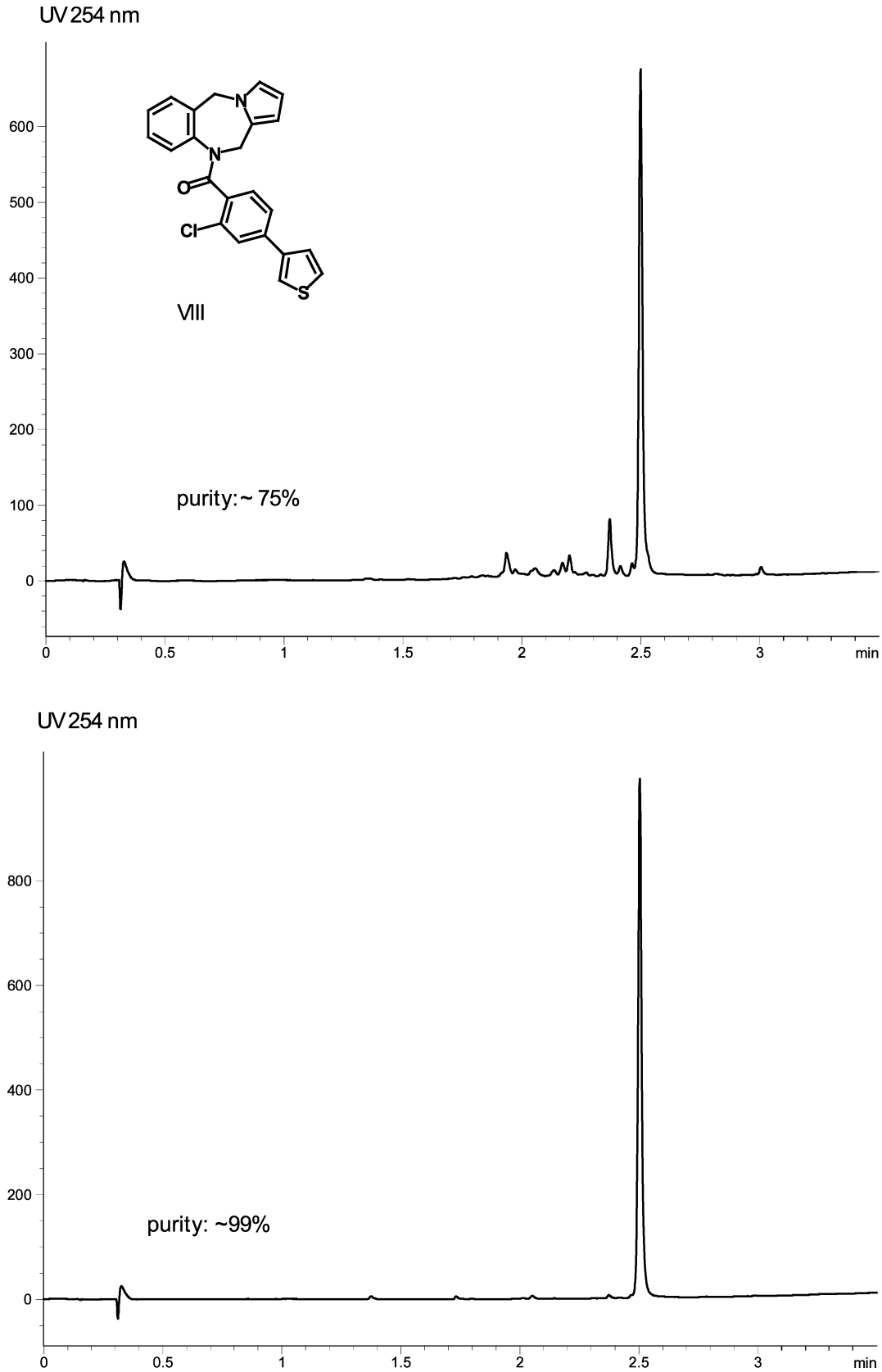

Figure 11. RP analytical LC/MS of compound **VIII** before and after purification (upper and lower traces, respectively), (see Experimental Section for conditions).

was purified (see above, discussion in Detection and Collection section and Figure 5).We found that recovery at this threshold was adequate using 10-mg injection level (~80%) and fairly high with 50-mg and 100-mg samples (~95% recovery). We also assumed that all members of the library, being of similar chemical structure, would produce ions of comparable intensity at the same concentration and a 20 000 units threshold could serve as a "universal" trigger point for samples of high (~90%) and low (~10%) purity.

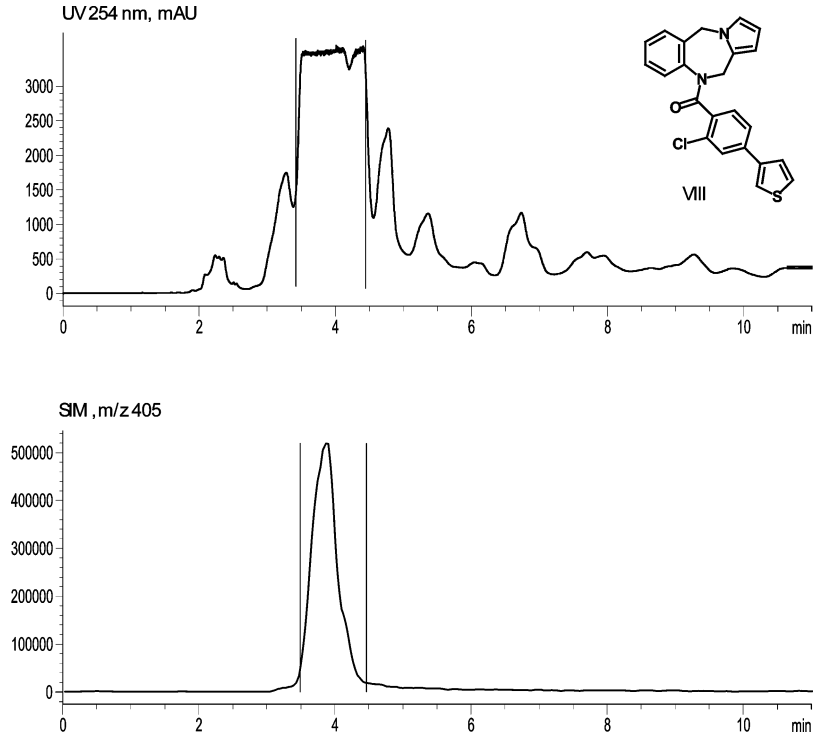

Figure 12. Automated purification of a 100-mg sample of compound **VIII** on a preparative Luna CN column, gradient 5−20% of solvent B in solvent A (Experimental), collection based on *m*/*z* 405 ion.

Use of Elevated Flow Rate for Purification of the Library Constituents. Preliminary experiments on purification of the library compounds were carried out at the flow rate of 20 mL/min; a flow rate that is optimal for 5 μm particles and column of this diameter (2 cm).[35,36] Eventually, the working flow rate was pushed up to 40 mL/min to achieve better throughput. A comparison of HPLC profiles obtained for a 100-mg sample of impure compound **X** at 20 and 40 mL/min is shown in Figure 6 (run at 20 mL/min with a cycle time of 4.66 s, maximum peak width of 2.5 min, delay loop volume ∼1.6 mL, the rest of the detection and fraction collection parameters being the same as in Experimental). The amount of material collected in both experiments was essentially the same: 29 and 26.6 mg for 20 and 40 mL/min, respectively (see Table 2 and details below).

Examples of Purification

Purification of Compound X. Isolation of compound **X** proved to be a challenging task and it was a real test of the purification system's ability to isolate fairly small amounts of pure materials from complex mixtures. In the original crude mixture the percentage of **X** was about 29% (Table 2; see analysis by NP and RP LC/MS; Figures 7 and 8, upper traces, respectively). After the first step of purification in the 5−20% gradient (Figure 9) a remarkable enrichment from 29 to ∼90% was achieved (Figure 7 and 8, lower traces, respectively) with yield of ∼26% (Table 2). The use of both chromatographic modes of analysis (NP and RP) gave us a high degree of confidence in the quality of the compounds obtained during purification.

One additional purification step under the same conditions was needed to achieve final purity of ∼98% for compound **X**.

Purification of Compound VIII. Compound **VIII** was found to contain about 25% impurities (using adsorption at 254 nm) when analyzed using both NP and RP LC/MS (upper traces of Figures 10 and 11, respectively). When subjected to purification on the Luna CN preparative column using the 5−20% gradient at 40 mL/min (Figure 12), 100 mg of **VIII** yielded ∼90 mg (Table 2). Post-purification analysis using NP and RP LC/MS confirmed the homogeneity of the isolated material (Figures 10 and 11, lower traces, respectively).

Conclusions

We've demonstrated that a commercially available mass-directed purification platform, based on NP HPLC using a preparative cyano column combined with APCI-MS detection, could be used successfully for quick purification of pharmaceutically relevant compounds slated for biological testing. This system was capable of purifying 23 compounds on a 100-mg scale in ∼8 h. The fixed parameters of two purification methods were programmed before the procedure using data on chromatographic retention and APCI ionization of the library compounds, which were obtained using analytical NP LC/MS in less than 3 h. Other polar bonded-phase columns (diol, amino) and mobile phases based on organic solvents other than those reported here, as well as isocratic methods of elution, may provide new capabilities for the fully automated purification of compounds of various chemical compositions on a preparative scale. We feel confident that this approach can serve as a fast and efficient alternative to conventional isolation methods based on RP HPLC and low-performance NP chromatographic techniques.

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