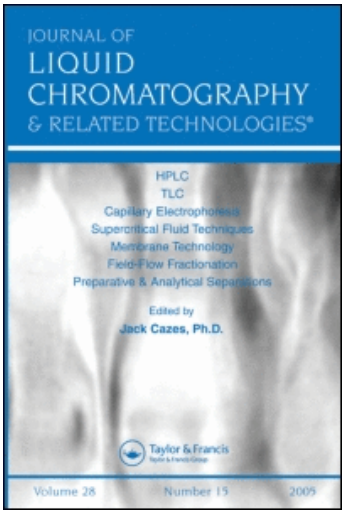

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Mass-Directed Normal-Phase Preparative HPLC with Atmospheric Pressure Chemical Ionization Detection

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Mass-Directed Normal-Phase Preparative HPLC with Atmospheric Pressure Chemical Ionization Detection

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ABSTRACT

A novel approach to auto-purification of a wide variety of organic compounds is described. It is based on normal-phase (NP) gradient high performance liquid chromatography (HPLC), performed on a 2 × 15 cm cyano column hyphenated with atmospheric pressure chemical ionization (APCI) source, and a single quad mass spectrometer (MS). A commercially available preparative HPLC–MS system, equipped with mass-directed fraction collection capabilities, has been successfully used for NP purification of neutral, acidic, and basic pharmacologically active compounds. Samples of 10–100 mg were chromatographed in gradients of methanol in hydrophobic organic solvents, and collected using generic chromatographic, detection and fraction collection experimental parameters. "Smart" collection—one component–one collection vessel—with 90–95%

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recovery, was achieved by controlling injection volume and gradient slope and by adding acetic acid and diethylamine to the mobile phase to keep peak elution volumes below 50 mL. Auto-collection of a solute was based on the main ion in its APCI–MS spectrum. The technique described has been also successfully used for chiral preparative HPLC applications and purification of non-UV-active compounds.

Key Words: Auto-purification; Preparative; HPLC; Normal-phase; Mass-directed; Cyano; APCI; LC–MS; Chiral; Non-UV-active compounds.

INTRODUCTION

Modern preparative high performance liquid chromatography (HPLC) techniques play an important role in synthetic organic chemistry. Despite tremendous progress towards production of new chemical entities and their evaluation as potential drug candidates (combinatorial and parallel synthesis), purification of those compounds on a scale of hundreds of milligrams still represents a challenging task for a medicinal chemist. As pharmaceutical companies move to enhance and upgrade their existing compound libraries, the necessity to produce more than a few milligrams of a pure compound, within the shortest period of time, dictates the use of highly specialized and efficient purification technologies. These techniques are mostly based on preparative reversed-phase (RP) HPLC methods in combination with electrospray ionization (ESI) and fraction collection based on mass spectrometric analysis.[1–4]

The low solubility of synthetic intermediates in aqueous media, and less than ideal processes used for sample recovery (samples sometimes are subjected to evaporation at elevated temperature under acidic conditions), are drawbacks associated with the use of RP preparative chromatographic methods. ESI is not an ideal ionization technique for many moderately hydrophobic synthetic intermediates, as it was initially designed for detection of molecules charged in solution.[5,6] As a result, "smart" collection (one component–one fraction) is not always possible with ESI–MS and other detection methods (UV, evaporative light-scattering) are needed.

Normal-phase (NP) preparative HPLC methods can provide a practical alternative to the RP approach, as they employ a variety of non-aqueous solvents on highly efficient polar columns (silica, cyano, diol) and provide excellent selectivity, better solubility for synthetic intermediates, good column loading capacity, and easy and safe solute recovery. The NP methods can be automated and used with the same preparative HPLC instrumentation as the RP ones. Gradient elution with washing and re-equilibration cycles, can

be used with cyano- and diol-type HPLC columns[7] simplifying method development and covering a broad range of solute polarities.

High retention, non-trivial method development, the inability to use gradients, and generation of significant amounts of flammable waste are associated with the use of bare silica columns for preparative HPLC.[8,9]

In this communication, we present a detailed report on previously, but briefly described,[10] novel approach to auto-purification of a wide variety of organic compounds. This approach is based on the use of an automated, mass-directed normal-phase preparative HPLC–MS system to isolate/purify ~10–100 mg of mixtures per run on a cyano column, using linear gradients of polar organic solvents in *n*-heptane or ethoxynonafluorobutane (ENFB)—solvent with similar chromatographic properties.[11] Several commercially available substances of various chemical nature were used as model compounds to investigate the feasibility and limits of applicability for such a technique.

EXPERIMENTAL

All compounds used in this study were purchased from Sigma-Aldrich (St. Louis, MO) or obtained from the Company's compound bank. Their structures are shown in Fig. 1. Samples were dissolved in methylene chloride, a mixture of methylene chloride and ethanol (1 : 1), or in dimethylsulfoxide (DMSO) at concentrations of up to 200 mg/mL, with the injection volumes varying from ~0.1 to 0.9 mL.

ENFB was purchased as 3M Novec™ Engineered Fluid HFE-7200 from 3M Company (St. Paul, MN). Its chromatographic properties are summarized in Ref.[11]. All other solvents were of HPLC grade and obtained from EM Science (Gibbstown, NJ).

A 1100 Series Purification System (Agilent Technologies, Palo Alto, CA), consisting of a preparative autosampler (G2260A, sample injection size: up to 1.8 mL), a gradient preparative pump (consisting of two isocratic G1361A pumps with flow rates of up to 100 mL/min at ~400 bar), an active (dynamic) splitter (G1968D) providing a variety of flow split ratios, a preparative fraction collector (G1364A with a tray featuring 40 collection vessels of ~50 mL collection volume), a UV-detector (G1315B), and an MSD mass spectrometer (MS) (Agilent, model G1946D) equipped with an atmospheric pressure chemical ionization (APCI) source. A small part of the preparative flow, after dynamic splitting, was mixed with a make-up flow of methanol–ethanol–acetic acid (500–500–1, v/v) provided by an additional isocratic HPLC pump (G1310A) and directed to the APCI source of the MS. The instrument was run using ChemStation software (version 9.01), along with Purify and active splitter control software packages (for hardware and software

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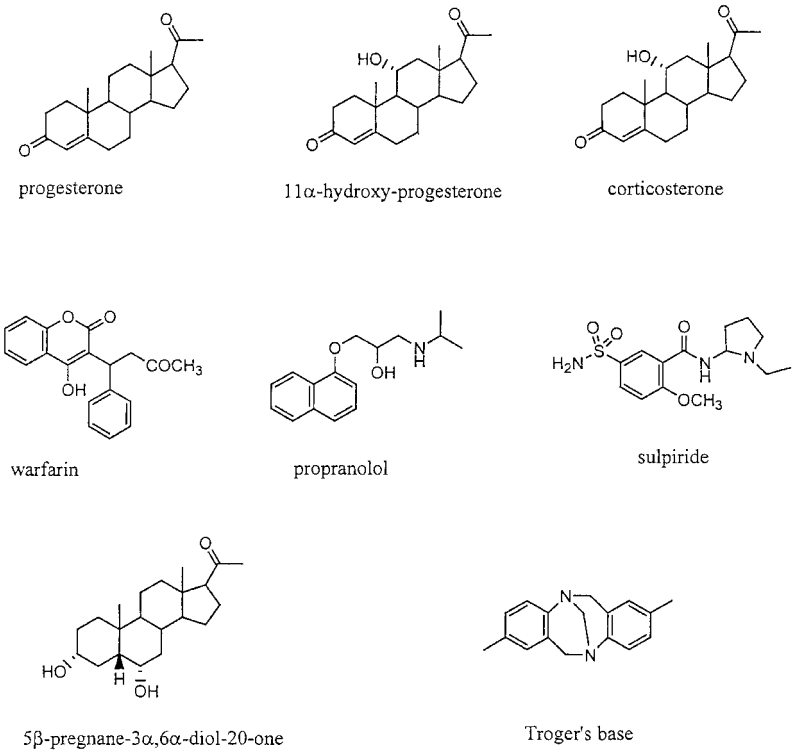

Figure 1. Structures of commercially available substances used as model compounds.

description see Ref.[12]). A custom high-throughput preparative LC–MS system using the same platform has been described recently.[2]

A Luna CN (2 × 15 cm, void volume ~38 mL) column packed with 5 μm particles (Phenomenex, Torrance, CA) was used for HPLC at 20 mL/min flow rate. Linear gradients of A in B were employed using two groups of solvents: I [A, *n*-heptane–methylene chloride (9 : 1); B, methylene chloride–methanol (8 : 2), 5–80% B in A in 20 min] and II [A, ENFB; B, methanol, 5–50% in 20 min]. Diethylamine or acetic acid (0.1%) was added to all solvents when basic or acidic compounds were chromatographed, respectively. Addition of 10% of methylene chloride was necessary to achieve reliable and accurate pumping of solvent A by the corresponding preparative pump.

The active splitter (Rheodyne, Cotati, CA) was set-up to provide approximately 1 : 20,000 split ratio. The column eluent was introduced into the APCI source operated under the following set of conditions: drying gas flow

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rate 41/min; nebulizer gas pressure 55 psi; drying gas temperature 300°C; vaporizer temperature 350°C; capillary voltage 6000 V, corona current 10 μA. Total ion current mass spectra were measured in a scan mode (220–600 amu) with the fragmentor set at 80, gain at 1, threshold at 250, step size at 0.1, peak width at 0.1 min, and cycle time at 1.89 sec/cycle.

The following parameters were used by the MS software to trigger fraction collection: single ion monitoring (SIM) of the ion(s) produced by the component(s) of interest: min and max peak width 0.1 and 2.5 min, respectively; slope 0, threshold 50,000–100,000. The information on the ionization pattern of the compounds studied was obtained using NP analytical LC–MS, as described previously.[13,14]

A delay time of ~0.13 min was measured to exist between the moment when a chromatographic peak leaves the splitting valve and when the MS "senses" signal produced by this peak, the preparative and make-up flow rates being kept at 20 and 0.5 mL/min, respectively. Accordingly, a piece of standard 1/16 in. OD PTFE tubing (volume ~2.6 mL) was introduced into the preparative flow path between the active splitter and the fraction collector to compensate for the delay and accurately collect chromatographic peaks.

RESULTS AND DISCUSSION

A system for automated HPLC purification of multiple samples, in principle, should be able to separate and selectively detect one, or several, components of interest using their unique physico-chemical properties, and collect component(s) into a single (preferably) collection vessel.

Potential problems one may encounter while trying to develop such a system include the sample load and injection volume size (both affect peak volume and resolution greatly), the nature of the injection solvent (must be compatible with the mobile phase), mode of detection (UV mode is not selective enough, while MS mode is sometimes too sensitive and the detector can be easily overloaded), column size (has to be kept constant to accommodate certain sample load), flow rate (needs to be kept constant to use generic gradients and successful MS detection), and gradient slope which has to be relatively constant to obtain reasonably constant peak elution volumes.

While working to develop a generic system for mass-directed NP preparative HPLC purification, we based our chromatographic approach on the recently described concept of using gradients of methanol and methylene chloride in hydrocarbon solvents,[7] or methanol in the hexane-like ENFB on cyano columns.[11] We found cyano-derivatized silica gel packing materials to be less retentive than silica and to have similar selectivity.

Cyano columns can be easily washed with strong solvent and then quickly equilibrated. The chromatographic conditions described can be applied to the efficient separation of a great variety of acidic, basic, and neutral compounds of various chemical nature: steroids, benzodiazepins, NSAIDs, tricyclic antidepressants, β-adrenergic blockers, and purines and pyrimidines.[11]

APCI Detection of Solutes Under NP Conditions

The ability to properly detect a chromatographic peak containing a component of interest and to trigger its collection is one of the most important features of any auto-preparative system. ESI–MS detection is widely employed when purification of combinatorial libraries is carried out in the presence of aqueous solvents,[1–4] while APCI can be used to ionize solutes separated under NP conditions.[15]

We found recently, that a great variety of neutral, basic, and acidic molecules can produce ions in the presence of such solvents (namely, methanol) when gradients of methanol in ENFB on cyano columns were employed for LC–MS applications.[13,14] The majority of neutral compounds of medium and low polarity were able to produce protonated molecular ions with good abundance and minimal fragmentation, the overall response frequently exceeding the response obtained for the same compound under ESI–LC–MS conditions. Basic and acidic compounds exhibited comparable response when studied under NP and RP LC–MS conditions using APCI and ESI detection, respectively.[14] These observations allowed us to investigate the ionization and fragmentation pattern of a compound of interest by NP analytical LC–APCI–MS. This information was then used to identify the solute by the MS after its elution from the preparative column, and trigger its fraction collection.

NP Preparative HPLC of Neutral Compounds with APCI–MS Detection

Keto- and hydroxy-pregnanes were used initially as model compounds to study chromatographic behavior of neutral molecules with moderate hydrophobicity, their APCI–MS response, and the ability of the auto-preparative system to recognize and initiate the fraction collection of a desired compound. Some of the first experiments to confirm the feasibility of our approach were injections of increasing amounts of 11-α-hydroxy-progesterone (MW 330) and successful auto-collection of the material injected (Fig. 2) after its gradient elution from the column using group I solvents (see Experimental). This compound was shown to produce a protonated molecular ion (m/z 331),

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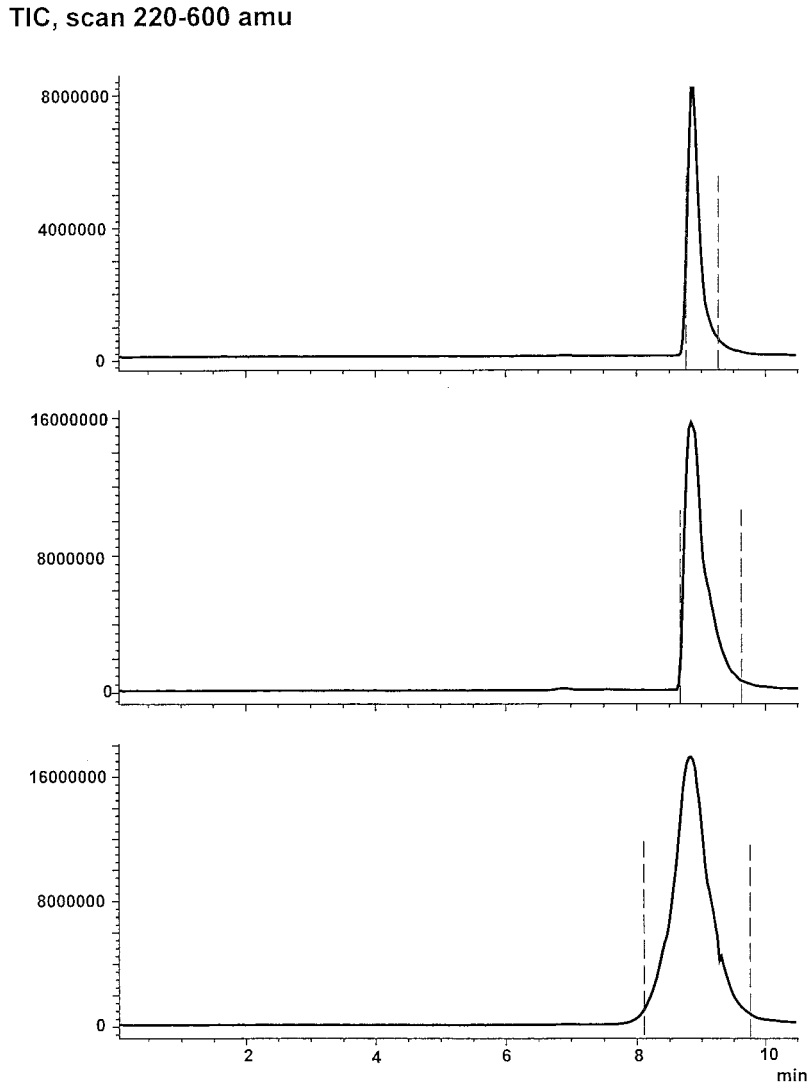

Figure 2. Mass-directed NP preparative HPLC of 11-α-hydroxy-progesterone. Linear gradient of B in A (5–80% in 20 min, solvent group I, Experimental). Flow split 20,000 : 1. Fraction collection based on a SIM (m/z 331) response, threshold 100,000, slope 0. 10, 50, and 100 mg injected in 0.1, 0.45, and 0.9 mL of methylene chloride–ethanol (1 : 1), respectively. Solid line, total ion current (TIC) response; vertical broken line, auto-collection start and stop.

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as the main ion under APCI–LC–MS analytical conditions with ENFB and methanol[14] and it behaved in the same manner in our system. When a fraction of preparative flow was mixed with methanol–ethanol–acetic acid make-up flow (Experimental), only a protonated molecular ion with m/z 331 was registered in the spectrum of the eluent, and the fraction collection was triggered with excellent efficiency (recovery 90–95% by weight). The system exhibited good chromatographic performance with peak elution volumes equal to ~0.25, 0.5, and 1 column void volume (~40 mL) for 10, 50, and 100 mg injected, respectively, and the APCI–MS response within 10–100 mg sample load range appeared to be linear (Fig. 3).

We investigated the ability of the system to isolate a main component from a mixture of several other components based on its molecular weight—a fairly common problem when a combinatorial chemistry or parallel synthetic methods are employed. A model mixture containing ~70% of 11-α-hydroxy-progesterone, ~15% of progesterone, and ~15% of corticosterone was prepared, and increasing amounts were subjected to preparative separation using the same conditions as in Fig. 2. Successful purification of the major component was achieved with loads up to 75 mg of the mixture injected (Fig. 4).

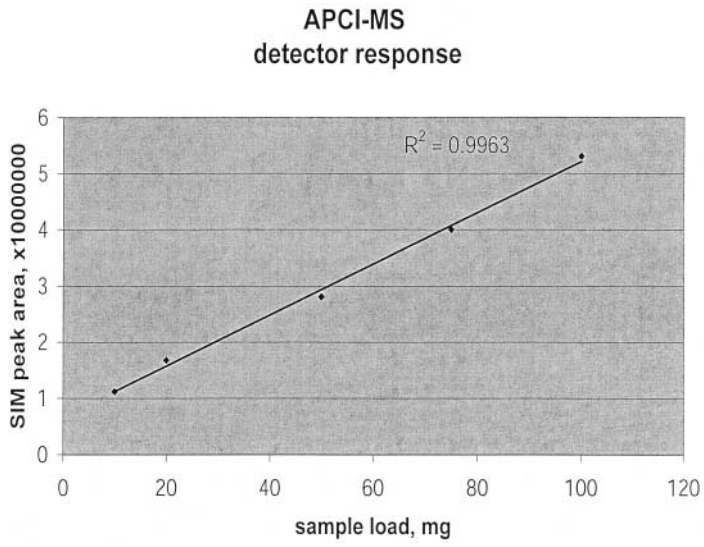

Figure 3. Linearity of APCI–MS detector response. Experimental conditions as in Fig. 1; 10, 20, 50, 75, and 100 mg of 11-α-hydroxy-progesterone were injected in 0.1, 0.18, 0.45, 0.7, and 0.9 mL, respectively. SIM (m/z 331) responses measured using standard ChemStation integration algorithm.

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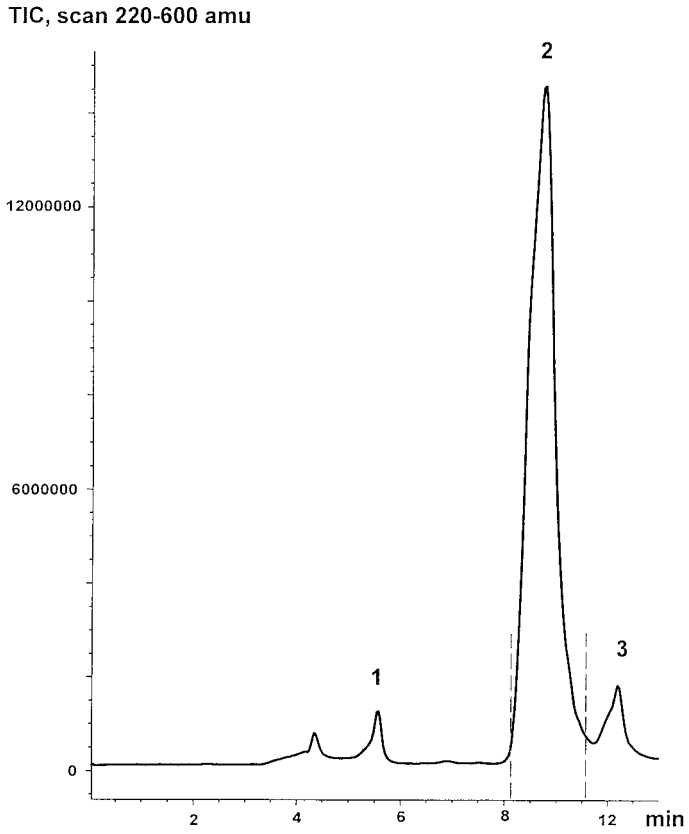

Figure 4. Isolation of 11-α-hydroxy-progesterone ($\sim$70%, peak 2) from its mixture with progesterone ($\sim$15%, peak 1) and corticosterone ($\sim$15%, peak 3). Conditions as in Fig. 1. Injected: 75 mg of the mixture in 0.75 mL.

NP Preparative HPLC of Acidic Compounds with APCI–MS Detection

A number of pharmacologically active compounds have acidic nature (e.g., warfarin, NSAIDs), and the ability to quickly synthesize and purify such compounds of similar structure plays an important role in structure–activity studies of potential drug candidates. Acidic compounds can be efficiently separated on cyano columns and gradients of polar solvents in hexane- and hexane-like mobile phases,[11] and detected under LC–MS conditions using an APCI–MS detector.[14]

We used warfarin as a model compound to demonstrate the feasibility of the NP chromatographic approach combined with APCI–MS detection in preparative HPLC applications. Preparative amounts of warfarin (~10–90 mg) were chromatographed on a 2 × 15 cm Luna CN column in a gradient of a mixture of methylene chloride and methanol (8 : 2) in heptane, with addition of 0.1% of acetic acid to both components of the mobile phase. APCI–MS detection was employed to detect the protonated molecular ion of warfarin (m/z 309) and trigger the automatic collection efficiently (Fig. 5).

The sample injection volume and the nature of injection solvent play an important role in the outcome of a preparative HPLC separation. The ability to dissolve significant quantities of a sample (~100 mg), in a relatively small amount of injection solvent(s) (1–2 mL), can be critical when purifying samples with varying degrees of hydrophobicity, as in combichem libraries.[2–4] DMSO appears to be a "universal" solvent to be used as an injection solvent, as the majority of organic materials have an excellent solubility in it. It is also inert and miscible with most common organic solvents. Its high viscosity, though, may prevent the proper mixing of a highly concentrated sample with less viscous components of a NP mobile phase and cause an injection band broadening and, eventually, chromatographic peak distortion.

We compared the peak shape and retention of ~100 mg of warfarin when injected in a minimal amount of methylene chloride–acetone–ethanol mixture (~1 : 1 : 1, injected volume 0.9 mL) and DMSO (0.3 mL), the latter producing a better retained and much more compact and symmetrical peak of the solute (lower trace, Fig. 5). Clearly, the injection volume played the major role in peak broadening/distortion process under chromatographic conditions used (2 × 15 cm column, 20 mL/min flow rate), as the same amount of warfarin injected in volumes larger than 0.5 mL of DMSO produced severely distorted peaks.

NP Preparative HPLC of Basic Compounds with APCI–MS Detection

Purification of basic compounds using NP chromatographic systems traditionally represents a challenging task for a medicinal chemist because of severe peak tailing on silica gel. We have shown, recently, that a great variety of such compounds can be efficiently separated on cyano columns, using gradients of a polar solvent in hexane and the hexane-like ENFB[11] in the presence of basic agents (triethylamine, diethylamine), as well as detected with an APCI source-equipped MS.[14] In this study, we applied the same chromatographic approach to auto-purify basic model compounds,

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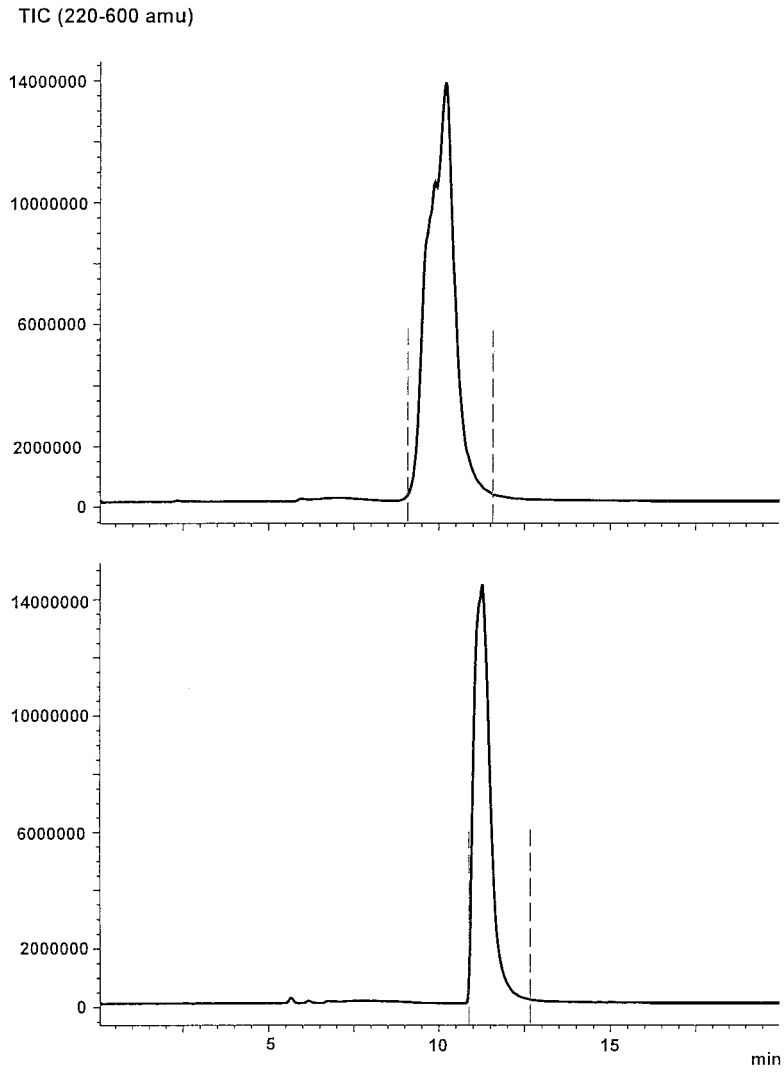

Figure 5. Effect of injection volume and injection solvent on mass-directed preparative HPLC peak shape and retention. Upper trace 90 mg of warfarin (free acid form, MW 308) injected in 0.9 mL of methylene chloride–acetone–ethanol (1 : 1 : 1); lower trace, 100 mg of warfarin injected in 0.3 mL of DMSO. Conditions as in Fig. 1 with 0.1% of acetic acid added to solvents A and B. Auto-collection based on $\{M + H\}^+$ SIM response (m/z 309).

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propranolol and sulpiride, in their free base forms on a 100-mg sample load scale. As expected, both compounds exhibited good chromatographic performance and were easily recognized and properly collected by the system (Fig. 6). The presence of 0.1% of diethylamine in the mobile phase (preparative flow) did not have any detrimental effect on the solutes' protonated ion production

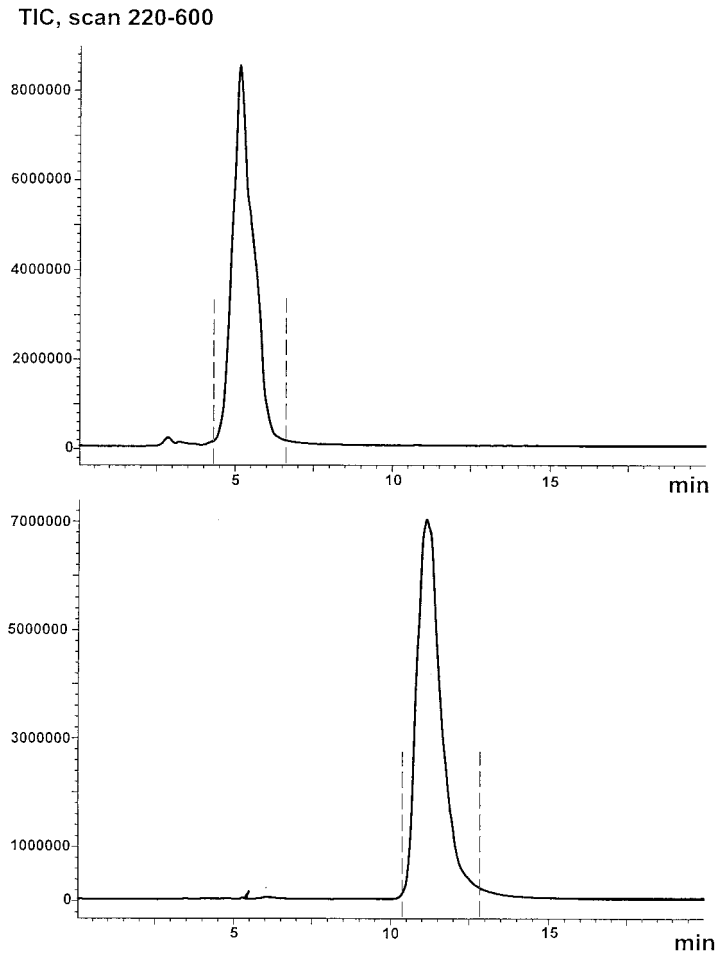

Figure 6. Mass-directed NP preparative HPLC of basic propranolol (MW 259, upper trace) and sulpiride (MW 341, lower trace). Both compounds injected as free bases (∼100 mg in ∼0.4 mL of methylene chloride–ethanol, 1 : 1 and DMSO, respectively). Conditions as in Fig. 1 with 0.1% of diethylamine added to solvents A and B. Autocollection based on protonated molecular ion SIM responses (m/z 260 and 342).

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inside of APCI source. This technique is currently being applied to the purification of a variety of basic compounds synthesized for several Discovery research programs. One such separation is shown in Fig. 7. The component of interest with MW 415, and possessing a basic dimethylamino group, was successfully isolated from the reaction mixture using gradient conditions with diethylamine added. The instrument's software was programmed to use its protonated molecular ion signal with m/z 416 for auto-collection of the reaction product.

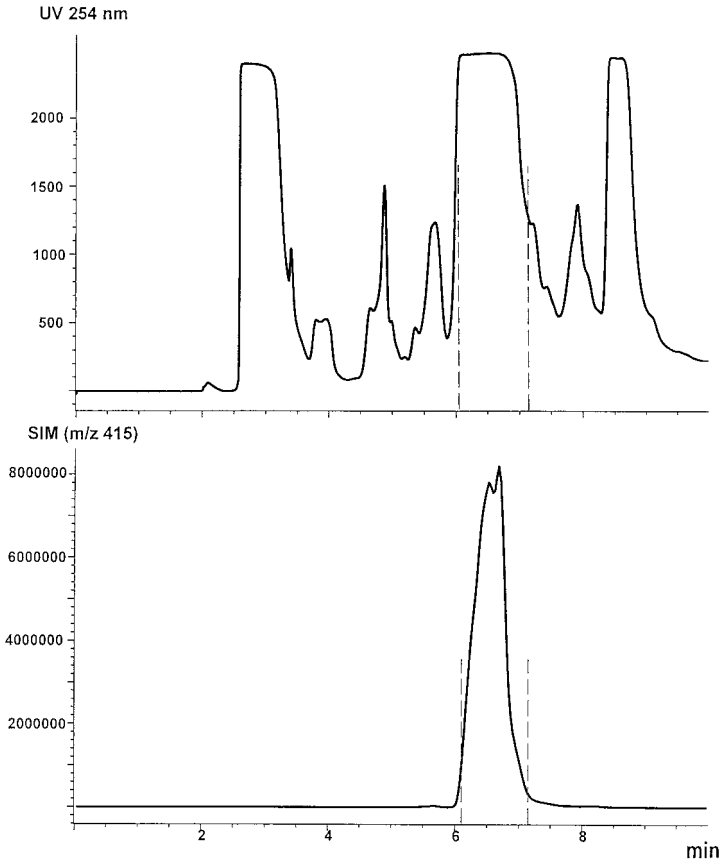

Figure 7. Purification of a basic synthetic intermediate (MW 414) from a crude reaction mixture. Gradient conditions as in Fig. 1. Total of ~140 mg were injected in 0.7 mL of methylene chloride. Upper trace, UV-detector response; lower trace, APCI–MS SIM response. Auto-collection based on protonated molecular ion SIM response (m/z 415).

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Mass-Directed NP Preparative HPLC of a Non-UV-Active Compound

A neutral steroid lacking a UV-chromophore—5β-pregnane-3α,6α-diol-20-one—was used to evaluate the ability of the auto-preparative system to detect and purify such compounds based solely on their ability to produce ions in the APCI source in the presence of organic solvents (Fig. 8). A 50 mg

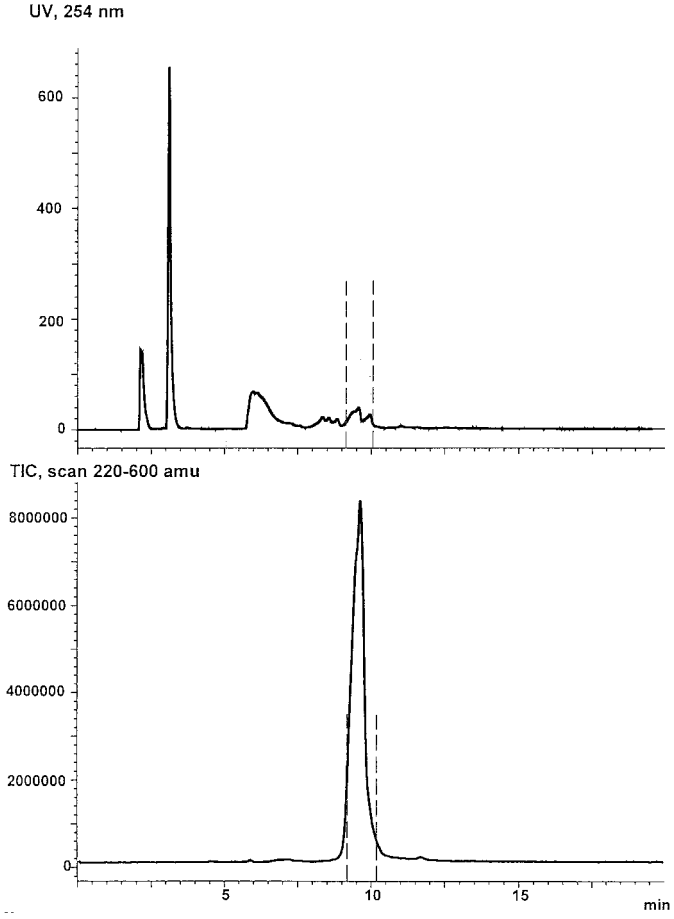

Figure 8. Mass-directed purification of 5β-pregnane-3α,6α-diol-20-one (MW 334). Separation conditions as in Fig. 1. Auto-collection based on $\{M + H - 2H_2O\}^+$ SIM response (m/z 299). Upper trace, UV-detector response; lower trace, APCI–MS TIC response.

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of the compound were injected onto a Luna CN column and eluted, as in Fig. 1. As expected, there was practically no response from a UV-detector at 254 nm wavelength, whereas the APCI–MS detector, after 20,000 : 1 flow split, was able to detect the chromatographic peak based on the main ion in its APCI spectrum {m/z 299, $(M + H - 2H_2O)^+$}, and triggered the fraction collector at the appropriate time. A 5β-pregnane-3α,6α-diol-20-one was collected with ~95% recovery (measured by weight before and after isolation).

Mass-Directed NP Chiral HPLC with APCI–MS Detection

As the majority of preparative chiral separations at this research facility are done using NP mode (either HPLC or SFC), we evaluated the APCI–MS detection as an alternative to traditional UV detection methods. A racemic model compound—Troger's base—was used in the experiments. We found, recently, that this racemate could be resolved analytically in a Chiralpak AD column using a gradient of methanol in ENFB with 0.1% of diethylamine added to mobile phase.[11] The same mass-directed set-up (described in Experimental) was used, with the Luna CN column being replaced with Chiralpak AD (2 × 25 cm) chiral column (Fig. 9). Troger's base (~50 mg, MW 250) was injected onto a column and the system's collection algorithm was set-up to monitor the response from its protonated molecular ion (m/z 251). Both enantiomers were successfully detected and automatically collected using essentially the same set of detection parameters, as the previous experiments with non-chiral samples. The fact that only a tiny portion of preparative flow diluted with the make-up solvent is used for ion recognition and subsequent mass-directed fraction collection, permits using the system with literally any combination of organic solvents, gradient or isocratic, and non-polar and polar ones. The system may also provide additional advantages of being able to detect and collect enantiomers lacking UV-active chromophores.

CONCLUSIONS

We have shown, that a non-customized, commercially available preparative HPLC instrument equipped with an APCI–MS detector can be used for mass-triggered auto-purification of neutral, acidic, and basic compounds, using a 2 × 15 cm cyano column and gradients of polar organic solvents in non-polar solvents. Generic chromatographic conditions, detection, and fraction collection parameters selected, allowed us to use the system in 10–100 mg per run sample size scale and collect solutes with peak volumes

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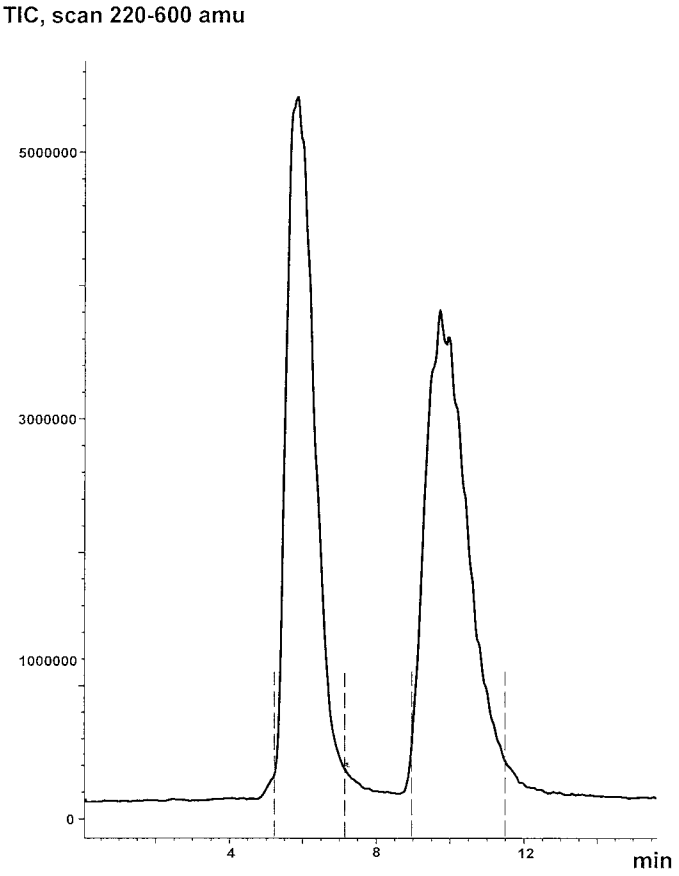

Figure 9. Isolation of Troger's base (50 mg injected in 1.8 mL of isopropanol, MW 250) enantiomers on a Chiralpak AD 2 × 25 cm column. Mobile phase: methanol gradient (5–50% in 20 min) in ENFB with 0.1% diethylamine, flow rate 20 mL/min (Experimental). Auto-collection based on protonated molecular ion (m/z 251). Other experimental conditions as in Fig. 1.

not exceeding 50 mL with 90–95% recovery. Polar solvents, including such a "universal" solvent as DMSO, were successfully used for sample injections as long as their volume stayed below 0.5 mL. The total injection volume, and not the physico-chemical properties of an injection solvent, appears to play a major role in the overall process of peak broadening and/or distortion. The system can be used to separate and auto-isolate main and/or minor

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component(s) from various mixtures, as the APCI–MS detector seems to provide a linear SIM response for components of interest in at least of 10–100 mg sample size range under experimental parameters used. "Smart" fraction collection (one component–one collection vial) has been achieved by controlling chromatographic parameters (injection size, gradient slope, and proper mobile phase additives) to keep peak elution volumes below maximal volume of the collection vessel (50 mL). The ability of the system to properly detect and collect components of interest when preparative and analytical flows are split in 20,000 : 1 ratio, allows the system to be used with virtually any mobile phase, gradient or isocratic, and polar or non-polar one. The APCI–MS detector may serve as a "universal" detector for preparative HPLC of organic molecules, complementing and possibly replacing conventional UV-detection technique as the broad range of organic compounds of different polarities and hydrophobicities produce strong ions in positive APCI–MS spectrum. The system described, provides additional advantages in separation and auto-collection of pure enantiomers in chiral preparative HPLC applications.

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