Development of a Custom High-Throughput Preparative Liquid Chromatography/Mass Spectrometer Platform for the Preparative Purification and Analytical Analysis of Compound Libraries

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Solution-phase parallel synthesis has had a profound impact on the speed of compound synthesis delivering relatively pure compounds (>80%) in short order. However, to develop structure activity relationships (SAR) for a compound series, each library member should preferably be >95% pure. Historically, achieving and quantifying such high-purity criteria for each library member proved to be the slow step for most lead discovery groups. To address this issue, significant modifications have been made to a commercial Agilent preparative LC/MS system to allow for the general mass-guided purification of diverse compound libraries. The custom modifications include (1) the "DMSO slug" approach for the purification of samples with poor solubility; (2) an active splitter to reduce system back-pressure, reduce the delay volume, and allow for a variable split ratio; (3) a sample loading pump for the quick purification of large, dilute samples; (4) a preparative column-selection valve to quickly change column selectivity or sample loading; and (5) an analytical injector with a separate flow path for crude reaction or fraction analyses.

Introduction

The need for expedient chemical lead discovery is crucial for the development of nascent medicinal chemistry programs and for the rapid delivery of safety assessment candidates to the clinic. In response, the pharmaceutical industry has established lead discovery groups whose mission is to synthesize analogue libraries on the basis of high-throughput screening hits. Each step of the lead discovery process, from screening to synthesis, is dynamic,[1] and every step must be evaluated independently and optimized.[2] Assay miniaturization coupled with robotics has revolutionized high-throughput screening, allowing for the evaluation of over 100 000 single compounds for a given biological target each week.[3] The speed of library synthesis has also dramatically increased by the application of solution-phase parallel synthesis whereby milligram quantities of single, "pure" (>80%) compounds can be obtained by the application of resin-bound or fluorous-tagged reagents/scavengers, solid-phase extraction, and automated liquid−liquid extraction protocols.[1,4−7] For many groups, the slow step in the discovery process centers on the purification and analysis of library members as a result of the industry trend toward producing high-quality (>95% pure), fully characterized compounds.[8,9]

In late 2001, Merck established the Technology Enabled Synthesis Division, which consisted of a lead discovery synthesis group and a purification group. Initially, the mission of the purification group was to only support lead discovery, but in time, all therapeutic areas within the medicinal chemistry department were actively being supported. As a consequence, the diversity of libraries (molecular framework, functionality, polarity, etc.) submitted for purification was enormous. In addition, both library size (12−160 members) and sample weights (1 mg to 1 g) varied widely. Moreover, initial (crude) sample purity ranged from under 5% to over 90%. A further complication concerns the dramatic variation in sample solubility and detector response due to the physiochemical properties of individual library members. The challenge for the purification group was to build a robust, versatile instrument to handle all sample purification requests, regardless of library/sample size or physical properties.

There are many techniques which can be used to purify drug discovery samples.[1,4−7,10−15] Because of the robust nature of the chromatography and past successes with UV-triggered fractionation, reversed-phase liquid chromatography was chosen as the base platform for sample purification. In addition, liquid chromatography (LC) has been shown to be readily automated and capable of producing compounds of high purity.[2,4,8,11,12,14−17] However, the capability of the LC unit to purify compounds in an automated fashion is highly dependent upon the configuration of the instrument and the physical properties of the sample. Samples with poor solubility can cause instrument clogging[8] or can go undetected and be lost if they ionize poorly (mass spectrometer detection)[9] or have a weak chromophore (UV detection). The advantage of automation can be lost if these problems lead to instrument failure. Our experience has been that while most of the current preparative liquid chromatography equipment performs acceptably when purifying samples with good characteristics, they often fail when challenged by difficult samples. Fortunately, these limitations can be overcome with instrument modifications and additions.

The detection system for preparative LC purification is an important consideration. The most common detectors for preparative liquid chromatography are UV and MS.[12] Many

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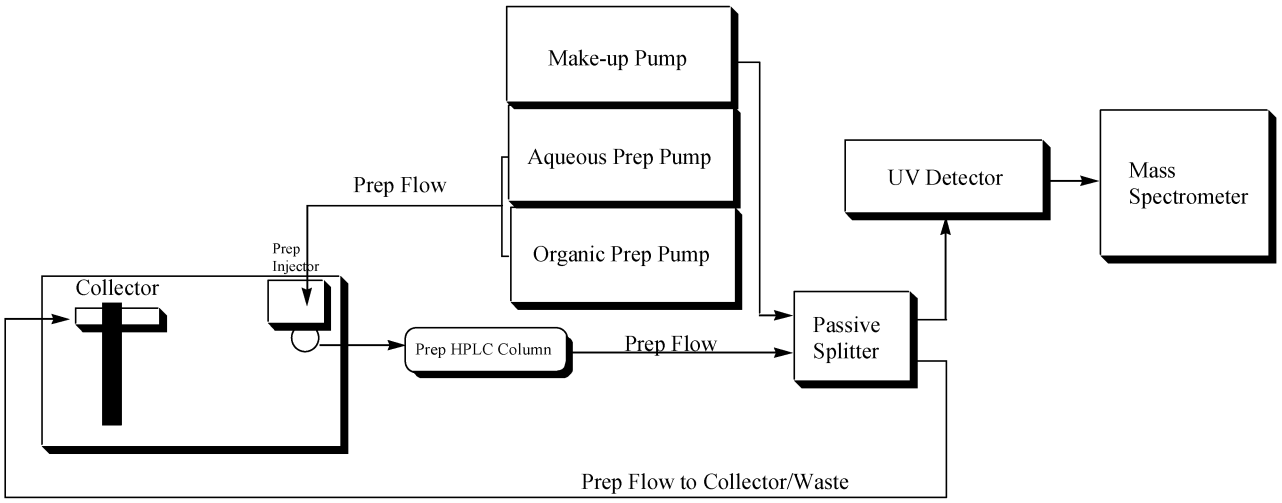

Figure 1. Configuration of original Agilent 1100 preparative liquid chromatography/mass spectrometer platform.

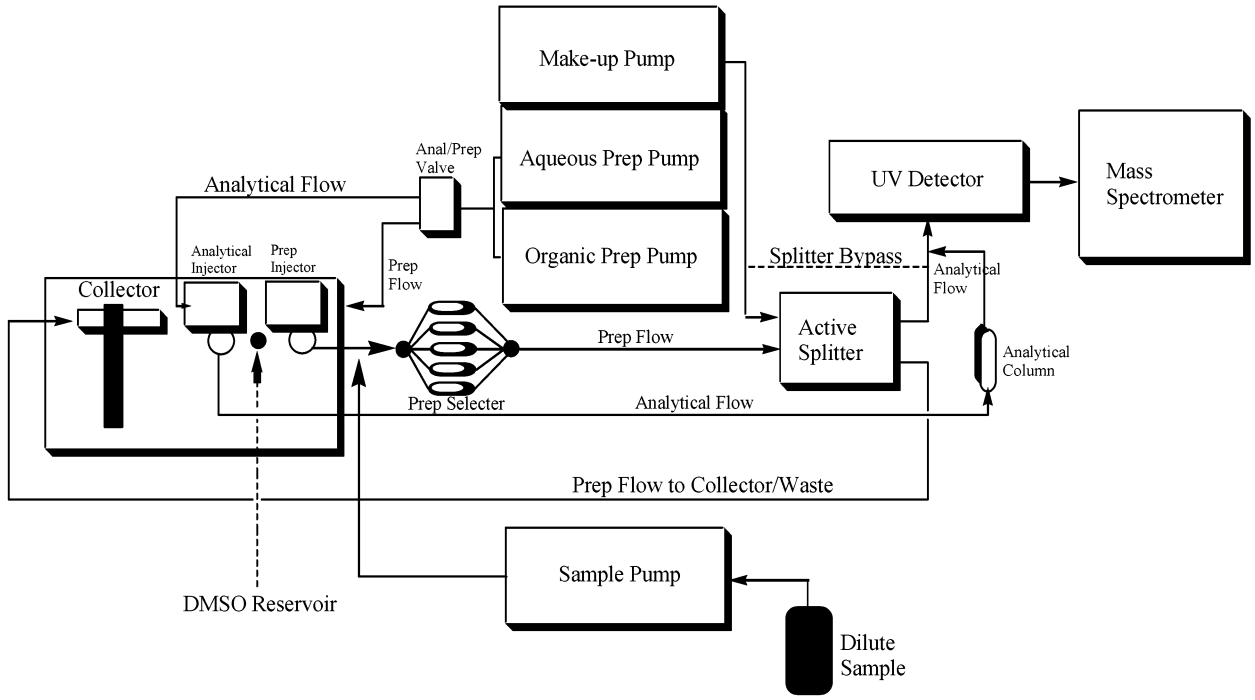

Figure 2. Configuration of Agilent 1100 preparative liquid chromatography/mass spectrometer platform following customization.

laboratories, including our own, have successfully employed preparative chromatography with UV detection for several years. Unfortunately, UV detection gives rise to the isolation of multiple components and multiple fractions/samples, which must be further analyzed to confirm identity and purity. Mass-guided purification is a more efficient method to isolate the compound of interest because the fractions are restricted to those containing the desired molecular weight.[10] For this reason, mass spectrometry (mass directed fractionation) was chosen as the method of detection/collection on which to design our purification platform. The associated UV (214 nm) signal was also collected, but not used to trigger fraction collection.

The goal for the platform is to obtain a purity of >95%, as determined by UV (214 nm) and ELSD in a single chromatographic pass. For months, several commercial preparative LC/MS units from a variety of vendors were surveyed. Ultimately, an Agilent 1100 Preparative liquid chromatograph/mass spectrometer mass-guided system was superior and chosen as the basic tool for general library purification. As alluded to earlier, the Agilent 1100 functioned reasonably well "out of the box", but with several modifications to the base system, vide infra, the versatility and robustness of the system increased significantly.

Experimental Section

The Agilent 1100 preparative LC/MS system was purchased from Agilent Technologies. A diagram of the original system is shown in Figure 1, and the customized unit is depicted in Figure 2. Solvent gradients were formed with a pair of Agilent 1100 Series preparative pumps capable of flow rates from <1 to 100 mL/min. The makeup and sample pumps were 1100 Series isocratic pumps. The flow rate of the sample pump was 1 mL/min with a mobile phase of 70/30 acetonitrile/water. The UV detector was an 1100 Series diode array detector. Sample injection/fraction collection was

Figure 3. The DMSO "slug" approach. Sample integrity is maintained by "sandwiching" the concentrated sample with a small volume (50 μL each) of air and neat DMSO. The DMSO prevents residual solvent in the flow path from direct interaction with the sample.

accomplished with an Agilent 220 Liquid Handler equipped with dual injectors. The preparative injector was configured with a preparative rotor and a 5-mL sample loop. The columns on the prep selection valve were 10 × 50 mm, 21 × 50 mm, and 30 × 50 mm Phenomenex Synergi 4-μm MAX-RP columns, a 30 × 100 mm Kromasil KR100-10-C18 column, and a 20 × 50 ODS-A YMC column. The analytical injector was configured with an analytical rotor and a 10-μL sample loop. The analytical column used was a 3.0 × 50 mm J'sphere H-80, 4-μm YMC with a run time of 4 min. The mobile phase for all chromatography was performed with gradients using acetonitrile/(0.1% TFA in water). The active inlet splitter, the analytical/preparative switching valve, and the six-column prep selection valve were all manufactured by Rheodyne and controlled by Agilent software. The active inlet splitter ratio used varied, depending upon the average mass spectrometer response for the series of compounds being purified. The mass spectrometer employed was an Agilent 1100 LC/MSD ESI System. The entire LC and MS systems are operated through Agilent CCMode and ChemStation software.

A typical sample protocol for library purification is as follows: A medicinal chemist prepares a sample library. The samples are dissolved in 1.0 mL of DMSO (filtering if necessary). The samples are chromatographed using an appropriately sized column on the Agilent mass-guided LC/MS. The compound of interest is collected on the basis of its exact mass. The pure fraction(s) is dried down in a Genevac Rotary Evaporator. An analytical analysis (either on-line or off-line) is performed to confirm purity. The pure solid is transferred to a tared, bar-coded vial and redried in a Genevac Rotary Evaporator. The vial is reweighed and returned to the medicinal chemist with all the obtained analytical data.

Results and Discussion

Maintaining Sample Solubility. The most basic challenge for any high-throughput chromatography system is maintaining sample solubility throughout the purification process. The nature of prep chromatography requires concentrated sample solutions. Since there is little or no physical data for the majority of samples requiring purification, either DMSO or DMF is used to dissolve all crude samples. In our experience, DMSO and DMF are the best solvents for solubilizing compounds with unknown physical characteristics.

However, solubilizing the crude sample represents only the first obstacle for maintaining sample solubility. Indeed, the sample must remain in solution until elution off the column. The problem of sample precipitation during chromatography must be addressed to have a robust system. Sample precipitation in the sample loop, injector rotor, or transfer tubing to the column can have disastrous results. The experience in our lab has been that if the sample arrives at the head of the column in solution, no blockages will occur downstream of the column. The rare precipitation that does occur postcolumn tends to be fine particles that easily pass through the wider bore (0.8 mm) tubing.

A previous preparative LC/MS system that we evaluated was plagued with sample precipitation problems. After loading the sample onto the sample loop, sample precipitation would occur prior to the column. The instrument manufacturer was unable to fix this problem. If care is not taken to maintain sample solubility throughout the chromatographic process, a preparative-mass spectrometry system can never be reliable, and sample throughput will be greatly reduced.

From our experience, the best way to eliminate in-line precipitation is by bracketing the injection. Specifically, the robustness of the Agilent preparative-mass spectrometry system was dramatically increased by utilizing the "DMSO slug" approach. In this approach, each injection is buffered by air gaps and DMSO (Figure 3) to maintain sample integrity. We have found that a volume of 50 microliters of air and DMSO works well for all preparative injections. Residual solvent in the sample flow path mixes with neat DMSO and not with the concentrated sample. Therefore, the concentrated sample remains in solution, since there is no direct interaction with the mobile phase. At our request, Agilent implemented this key CCMode software modification so that samples that are insoluble in water and even insoluble in acetonitrile are routinely purified without incident.

Active Splitter. When using Mass Spectrometry as the trigger for fraction collection, the sample flow must be split. The majority of the flow passes through to the fraction collector, while only a very small portion of the prep flow is diverted to the mass spectrometer to trigger sample collection. Traditionally, flow splitting has been performed with a passive splitter (Figure 4). A passive splitter maintains a constant flow split with a tee, that is, one outlet connected to a short piece of capillary tubing and the other outlet connected to a much longer piece of wider bore tubing that acts as a delay loop.[18] The majority of the flow passes through the wide bore tubing. This type of splitter has limitations. The split ratio cannot be changed. The delay loop built into the splitter causes back-pressure and peak-broadening due to diffusion. A back-pressure differential between the prep flow outlet and the analytical flow outlet must be maintained, usually by adding a piece of narrow bore tubing to the prep flow outlet side of the splitter. At higher flow rates, the narrow bore tubing is removed to decrease system back-pressure. Thus, a change in flow rate can require a change in the system hardware.[18]

To circumvent these limitations, the passive splitter has been replaced with an active splitter.[18] The Rheodyne active splitter valve oscillates, taking an aliquot from the prep stream and placing it in the mass spectrometer stream. Since there is no need for narrow bore tubing to maintain a pressure differential, the overall system back-pressure has been reduced, and flow rates from 6 to 60 mL/ min are easily

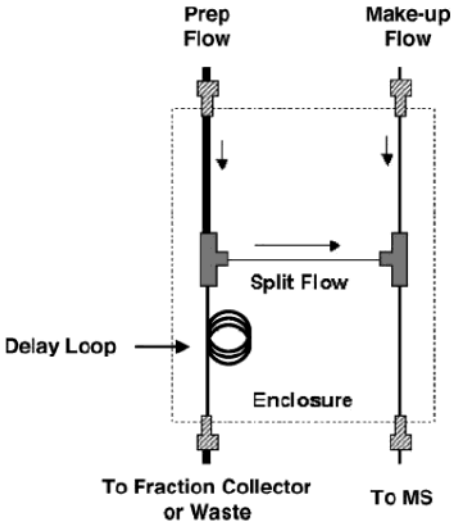

Figure 4. Schematic diagram of a passive flow splitter configured with a makeup or secondary carrier stream. The fractional split is introduced to the secondary stream in order to both dilute and speed its transport to the detector. The delay loop volume is adjusted to compensate for the time needed for a portion of the primary stream to cross the split flow transfer tube. Large split ratios require narrow transfer tubing, and thus, large delay volumes are used (reprinted from ref 18 with permission).

adjusted without any change in system hardware. The active splitter splits the analytical and prep flow in real time. There is no time delay after the flow is split so there is no need for a delay loop; hence, excess tubing is eliminated. In addition, the Agilent system can very quickly start fraction collection following peak detection since the detector communicates directly with the fraction collector. As a result, the time required by the software to trigger fraction collection has become negligible (0.01 min). Both of these factors have substantially reduced the amount of time the sample spends in coiled tubing postcolumn. Significantly, the time post-detection has been reduced from 0.42 min with our previous preparative-mass spectrometry system with passive splitting to 0.06 min (both at 25 mL/min) with an active-splitter-equipped Agilent system. Through the first 12 months of its use, the active splitter has performed well with no down time.

With multiple flow paths and an adjustable frequency, the splitter has a 10^3 range of split ratios. Using the Agilent ActSplit software, the split ratio can be adjusted while maintaining a high sampling frequency. This has been valuable for our lab to collect samples that do not ionize well. For samples with poor ionization, our normal split ratio (2083:1) is lowered (625:1) to increase detector sensitivity. Importantly, this allows samples with poor ionization to be successfully purified by mass-triggered fractionation.

One concern with the active splitter is the production of a segmented flow. The analytical flow "split" occurs every 0.5−2 s, depending upon the chosen active splitter frequency. The interruption in flow produces a "sawtooth" appearance in the mass spectrometer chromatogram (Figure 5A). To control the "sawtoothing" effect, we have introduced a partial bypass of the splitter (Figure 6). The makeup flow to the splitter is partially diverted around the splitter. Narrow-bore tubing (0.005 in.) was used for the bypass to ensure good flow through the splitter so that the valve groove was washed consistently, with the bypass negating the pressure pulsing. There is no change in signal intensity, but the "sawtoothing" has been greatly reduced (Figure 5B).

Sample Pump. An analytical pump has been added to the solvent path directly before the preparative column (Figure 2). This pump functions as a sample pump for reinjection of large volumes of dilute sample. During normal sample loading through the injector, the volume that can be loaded in a single injection is limited to the volume of the sample loop. The sample loop capacity becomes a limitation when large volumes of dilute sample need to be purified, such as for the repurification of impure fractions. Since this modification bypasses the sample loop, there is no limitation to the volume that can be loaded directly onto the column with a sample pump. As a result, loading is volume-independent. The aqueous and sample pumps are operated in unison during loading, and the precise flow composition of these pumps is dependent upon the relative solvent strength of the sample solution. For example, a sample solution high in organic content requires a higher aqueous pump flow rate. Although this type of loading does not work

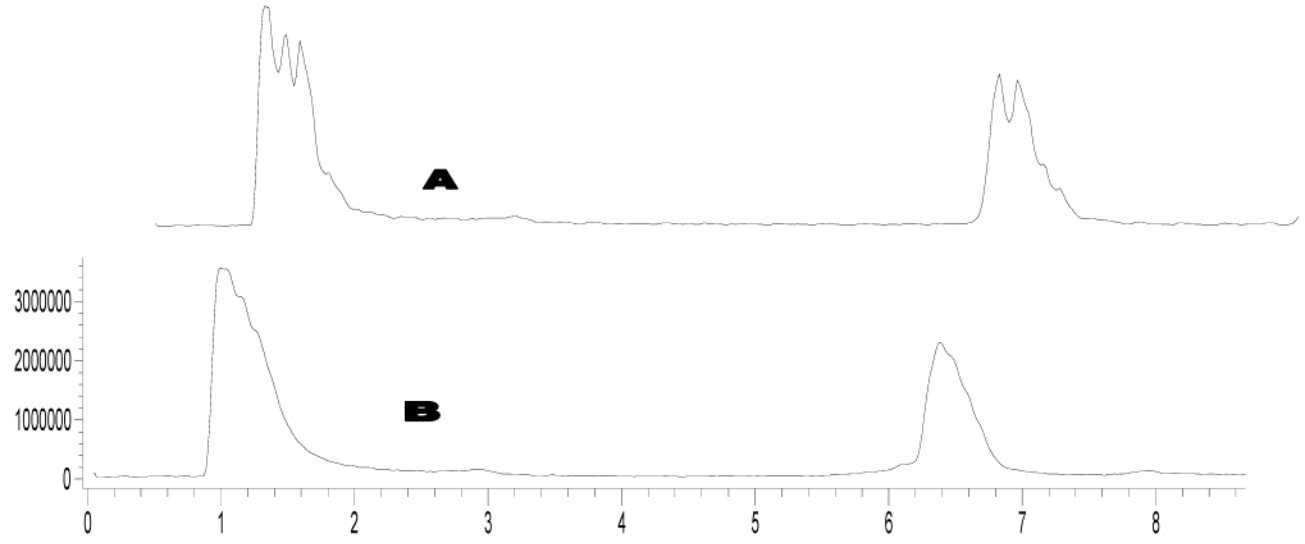

Figure 5. The total ion current of a sample injected twice without (A) and with (B) the splitter bypass configuration (Figure 6).

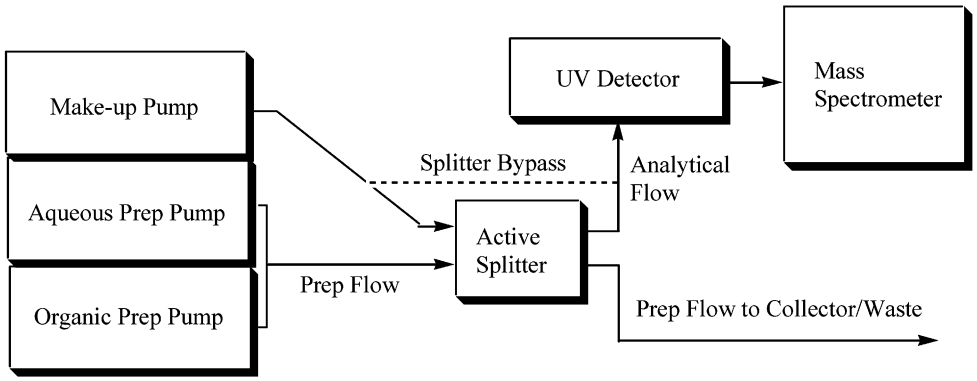

Figure 6. Flow diagram showing the splitter bypass configuration. The makeup pump flow is partially diverted around the active splitter to greatly reduce the "sawtoothing" effect.

well for concentrated sample that can precipitate, dilute sample solutions can be quickly loaded directly onto the column without the need for a lengthy sample concentration step.

Preparative Selection Valve. To adapt to different sample sizes, it was important to be able to switch between columns quickly.[17] Instead of constantly changing columns manually, a Rheodyne preparative selection valve was incorporated into the system (Figure 2). The six-position valve was fitted with a 10-mm column, two 20-mm columns, two 30-mm columns, and a column bypass. The amount of sample to be purified dictated the diameter of the column selected (Figure 7). After evaluating columns from several manufacturers, we chose the Phenomenex Synergi 4-μm MAX-RP column as our column of choice. The additional positions on the selection valve were fitted with more reversed-phase columns from other manufacturers. As part of the ongoing refinement to our process, we continue to evaluate new columns. The column selection valve provides a convenient way to split a library purification between two different columns. The "apples to apples" comparison is important in making informed decisions on column performance.

An additional benefit of being able to quickly switch between columns from different manufactures is to provide chromatographic selectivity. Occasionally, samples do not meet our purity requirements because of impurity coelution during purification. These samples could be successfully repurified if chromatographic selectivity were changed. Although changing the mobile phase might be the most effective way to change selectivity,[20] it is not conducive to a high-throughput operation. Therefore, our platform alters chromatographic selectivity by changing to a column from a different manufacturer. We routinely rechromatograph impure samples, via our sample pump, onto a different column with excellent results. The column selection valve expedites this process.

Analytical Analysis. Following purification, each sample must be analyzed to confirm identity and purity. Initially, this analysis is performed by analytical LC/MS. It would be convenient to perform this analysis on-line with the preparative system. However, the system was optimized for preparative chromatography. The large-bore tubing and a 5-mL sample loop make analytical chromatography impractical.

To obtain high-quality analytical data with the present platform configuration, a separate sample flow path for analytical analysis was required.[11] An analytical/preparative selection valve and a separate analytical injector were added to the platform. By isolating the analytical components of the system, wide-bore tubing is not required. The entire flow path consists of narrow-bore (0.010-in.) tubing. The analytical injector and analytical column are teed into the solvent path directly after the active splitter (Figure 2). Splitting the flow is not necessary at an analytical flow rate (1.2 mL/min), and the sample flows directly to the UV and mass detectors. When instrument time is available, fractions are directly subjected to analytical analysis from their prep collection tubes (Figure 8). The CCMode software provides the flexibility to selectively choose fractions for analysis or to automatically analyze all fractions. A second option is to simply prepare HPLC vials for analysis. Our Agilent 220 Liquid Handler can be configured with a rack that holds HPLC vials. Preparative fraction and HPLC vial analyses are generally run in an unattended overnight operation.

The addition of the analytical path also allows for analytical analysis prior to purification. That analytical data can be used for optimizing the preparative method. We are pursuing this approach as part of the next step in the evolution of our purification process.

Conclusion

Our Agilent preparative liquid chromatography/mass spectrometer worked reasonably well "out of the box" for routine soluble samples. The limitations of this system and other prep/mass spectrometers systems currently available is that they are limited in their ability to handle samples with poor water or acetonitrile solubility or poor ionization. To increase the robustness of the system and to increase its versatility, several key modifications were incorporated to the base platform.

The robustness of the system has been greatly enhanced by incorporating software to allow for "DMSO slug" injections. By buffering the concentrated sample between two portions of DMSO, sample solubility is maintained. Even samples with poor solubility in water or acetonitrile can be chromatographed in this manner, reducing instrument down time due to in-line sample precipitation. The actual number

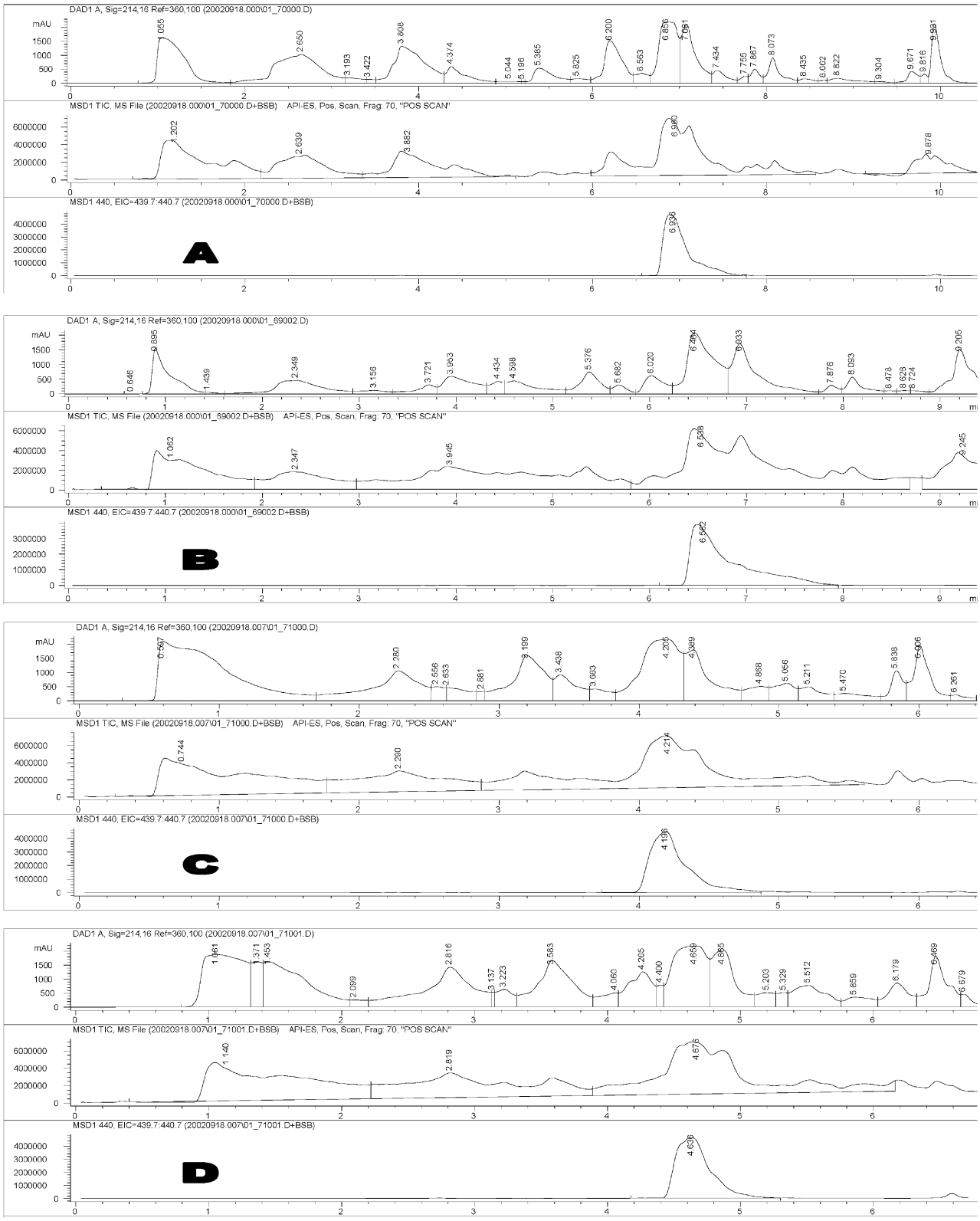

Figure 7. The prep selection valve allows for convenient switching between several columns. In each example, a 150 mg/mL sample was injected. The top trace of each set is at 214 nm, the middle trace is the total ion current, and the bottom trace is the extracted ion of interest ($MH^+ = 440.2$). (A) A 45-mg injection on the 10 × 50 mm Phenomenex Synergi 4-μm MAX-RP column. The gradient was performed with acetonitrile/(0.1% TFA) water from 0 to 50% acetonitrile at 8 mL/min over 8.5 min with a run time of 10.5 min. (B) A 165-mg injection on the 20 × 50 mm Phenomenex Synergi 4-μm MAX-RP column. The gradient was performed with acetonitrile/(0.1% TFA) water from 0 to 50% acetonitrile at 25 mL/min over 8.3 min with a run time of 9.4 min. (C) A 290-mg injection on the 30 × 50 mm Phenomenex Synergi 4-μm MAX-RP column. The gradient was performed with acetonitrile/(0.1% TFA) water from 0 to 50% acetonitrile at 55 mL/min over 5.3 min with a run time of 6.4 min. (D) A 290-mg injection on the 30 × 100 mm Kromasil KR100-10-C18 column. The gradient was performed with acetonitrile/(0.1% TFA) water from 0 to 50% acetonitrile at 55 mL/min over 5.3 min with a run time of 6.8 min (see ref 19).

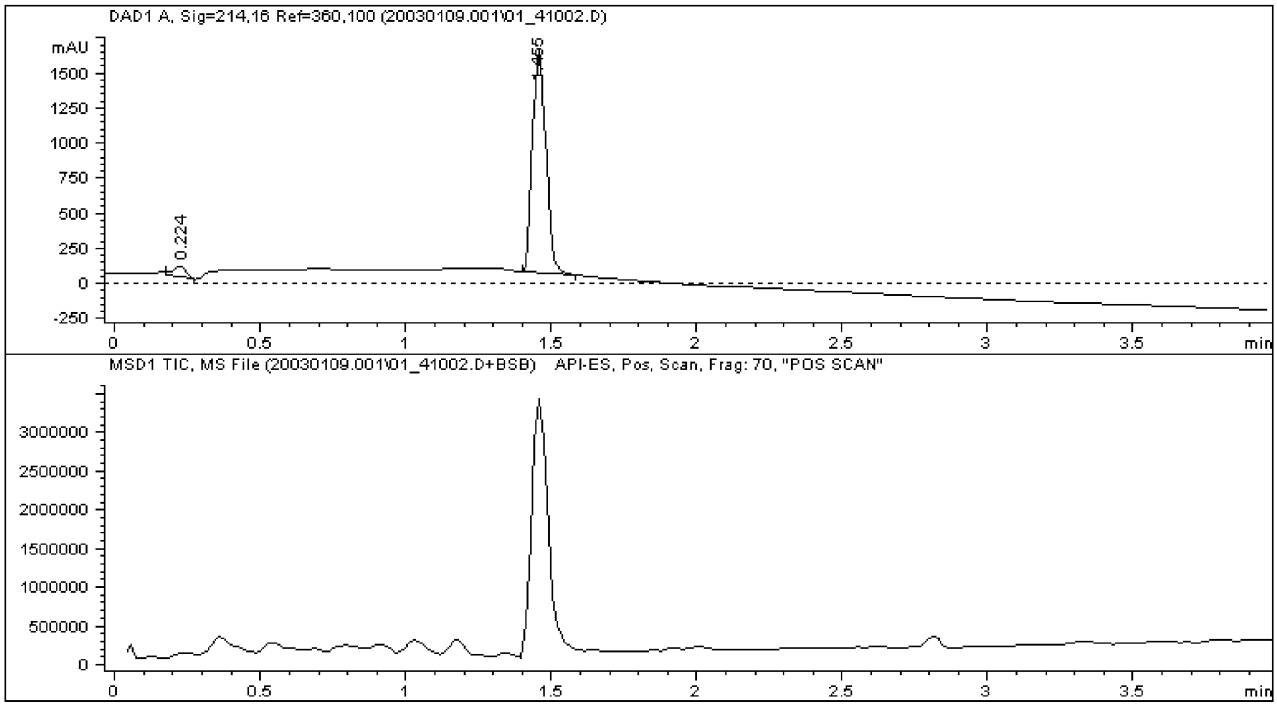

Figure 8. Typical chromatogram of a purified fraction using the analytical flow path of the Agilent 1100 preparative liquid chromatography/mass spectrometer platform. The top trace is at 214 nm, and the bottom trace is the total ion current. The gradient was performed with acetonitrile/(0.1% TFA) water from 4 to 100% acetonitrile at 1.2 mL/min over 3.5 min with a run time of 4.0 min. The analytical column used was a 3.0 × 50 mm J'sphere H-80, 4-μm YMC.

of down time occurrences we have experienced following our modifications has decreased from 1−2/week to 1−2/6 months.

The versatility of this system has also been increased by further modification. The active splitter we now use has an adjustable split ratio. The relative sensitivity of our mass spectrometer can be changed to match the average mass spectrometer response for the particular sample library being purified. The split ratio is increased for sample libraries with strong mass spectrometer responses to prevent the detector from overloading. Conversely, the split ratio is decreased for sample libraries with weak mass spectrometer responses to allow for sample detection. The splitter bypass has solved the problem of "sawtoothing" created when using an active splitter. A sample pump eliminates the need for a lengthy drying-down process for the repurification of large dilute samples. These samples can be pumped back onto the column without the constraint of sample loop size. The prep column selection valve allows for a convenient way to change between many different columns. This facilitates the chromatography of different sample sizes and a change in column selectivity. Finally, the addition of a separate flow path for analytical analysis produces quality analytical chromatograms. On-line analysis of fractions and HPLC vials eliminates the need to transfer samples from the preparative instrument to another analytical instrument to assess purity.

Two modified systems are currently in operation, affording excellent mass recoveries (70−95%) and purities (>95%) with a capacity to purify ∼6 samples/h per instrument. Additional modifications and refinements to these custom Agilent units are under investigation and will be reported in due course.

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