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Automated analytical/preparative high-performance liquid chromatography–mass spectrometry system for the rapid characterization and purification of compound libraries

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Abstract

A fully automated analytical and preparative HPLC–MS system has been developed and applied to the characterization and purification of compound libraries derived by parallel synthesis. Our automated LC–MS system incorporates fast, reversed-phase C_{18} HPLC (5–10 min analyses) and electrospray ionization mass spectrometry (ESI–MS). Post-data acquisition purity assessment of compound libraries is performed automatically using applescripts. Compounds falling below a threshold level of purity ($\ll 90\%$) are subjected to automated on-line preparative HPLC–MS (i.e., PrepLCMS) purification. PrepLCMS is a method that we developed and is the first mass spectrometry-based system that permits automated and rapid purification of multimilligram quantities of compound libraries using a mass spectrometer to 'signal' fraction collection. The method utilizes real-time mass spectrometric ion signals to trigger fraction collection and only the mass(es) of the compound(s) identified by the user in the data acquisition method are collected. This one-sample–one-fraction format means that 'batches' of compounds can be purified without the need for excessively large fraction collector beds. Further, this eliminates the need for post-purification analysis or pooling of fractions collected, typically associated with other preparative purification techniques. This technique was designed for use by the chemist in an 'open access'-like environment, and has been successfully implemented for automated and unattended batch purifications of large numbers of compound libraries derived by parallel synthetic strategies. The results for several compound libraries, synthesized and purified at the multimilligram level by automated PrepLCMS are presented.

Keywords: Combinatorial chemistry; Preparative chromatography; Parallel synthesis; Mass spectrometry

1. Introduction

Both pharmaceutical and biotechnology industries have embraced the challenge in recent years of developing newer, faster and more efficient ways to synthesize and screen pharmaceutical compounds to generate drug candidates against the steadily increasing number of biological targets being identified as a direct result of the human genome initiative. The traditional one compound–one screen approach has been replaced with high throughput methods (i.e., screening large numbers of compounds against multiple targets) as a way to rapidly identifying 'hits' and developing them into promising lead candidates. Advances in high throughput screening have come about principally through the implementation of automation/robotics into the laboratory. Advances in high throughput synthesis have resulted from the emergence of combinatorial chemistry. The advent of combinatorial chemistry has provided the medici-

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nal chemist with new 'tools' to accelerate the synthesis of pharmaceutical compounds, most notably, the ability to synthesize large 'libraries' of compounds through automated parallel synthesis [1,2] and split-couple-recombine (or split/mix) methodologies [3,4].

Split-couple-recombine methods have been used to produce large, diverse libraries of compounds [5–8]. A caveat to the split-couple-recombine approach, however, is that the enormous size of the libraries precludes their ability to be characterized fully and accurately. Smaller libraries derived by this approach have been amenable to analytical characterization [9–13], and two additional issues associated with the split-couple-recombine approach have been identified. They are: (i) rates of reaction will differ for members of a given monomer set, and hence, the concentrations of the individual components within the mixture will not be equivalent and (ii) side-products will be generated invariably, complicating the deconvolution process and interpretation of data, as well. Consequently, the ability to draw meaningful and reliable inferences from the structure–activity relationship (SAR) data of the individual screens is compromised. Converting 'hits' identified from the initial screen into 'leads' employing this strategy alone, is also a formidable task, as extensive iterative deconvolution strategies are required to 'decode' activities from complex mixtures [14,15,9]. One-bead, one-compound [16] and encoded or 'tagged' libraries have presented alternatives to iterative deconvolution [17–22].

Recently, 'split-couple-recombine' methods have been complemented with automated, parallel high-throughput synthesis (HSS) of discrete compounds as a means for generating optimally designed combinatorial libraries. HSS strategies permit the composition of individual components within the library to be assessed and therefore permit meaningful SAR to be gleaned and hypotheses derived about ligand binding from initial screening data with a high degree of confidence.

Smaller, optimally-designed libraries have been synthesized extremely efficiently and rapidly using automated, solution and solid-phase parallel HSS strategies. However, not all synthetic steps proceed equally efficiently and the yields and purities can vary dramatically. Consequently, the need has been created for developing exquisitely rapid methods for the characterization and purification of combinatorial libraries. 'Open-access' or 'walk-up' mass spectrometry provides the medicinal chemist with automated tools for the rapid qualitative characterization of pharmaceutical compounds [23–25] utilizing flow-injection electrospray ionization mass spectrometry (ESI–MS). This technique has been shown to be particularly useful for assessing synthetic intermediates, although it is not capable of providing an accurate assessment of compound purity [26,27].

Automated, semi-quantitative assessment of combinatorial libraries is most readily accomplished coupling HPLC with UV detection and ESI–MS [28,29,7] and more recently, incorporating evaporative light-scattering detection [30]. Traditional HPLC methods, however, do not provide the throughput necessary to handle the size and complexity of combinatorial libraries. Recently, rapid HPLC methods have been introduced for the analytical characterization of compound libraries [31,32]. Short columns and very fast gradients (typically 3–10 min per sample) have been used to facilitate analysis and the high-resolution separations afforded by these columns have permitted their widespread application in combinatorial chemistry.

The ability to characterize compound libraries generated by parallel synthesis has often revealed that the compounds generated by this method are not suitably pure for biological screening. Purification strategies that have been employed traditionally to purify multimilligram quantities of organic compounds include crystallization and preparative TLC. Techniques capable of being fully automated, such as preparative HPLC, are gaining popularity for the purification of combinatorial libraries. Typically, when using automated preparative HPLC, fraction collection is initiated upon a threshold UV signal being observed. This leads invariably to the collection of multiple tubes per sample, and hence creates additional issues, including: (a) the need to use very large fraction collector beds when purifying large numbers of compounds and (b) the need for secondary analysis (typically by Flow Injection–MS or HPLC–MS using electrospray ionization), to identify the appropriate fraction(s). When purification of large numbers of compounds is required, typical of most parallel syntheses, this approach can lead to unnecessary losses of very valuable time.

To facilitate the purification of compound li-

braries, we have developed a fully automated, rapid and novel method for the preparative scale purification of combinatorial libraries. The method, we term, PrepLCMS, incorporates fast chromatography and 'intelligent' fraction collection using electrospray ionization mass spectrometry. The power of the technique lies in that only the mass of the compound of interest within a given mixture needs to be identified in order for unattended, automated preparative purification to proceed. Fraction collection is initiated upon a real-time threshold reconstructed ion current signal being observed for a particular *m/z* input value. Thus, only one fraction is collected per sample. This 'one sample–one fraction' paradigm permits 'batches' of compounds to be processed without the need to resort to excessively large fraction collection beds. Further, no post-purification screening and pooling is required to identify the purified fraction of interest. We demonstrate that PrepLCMS can be performed fully unattended and overnight with the capacity to purify multimilligram quantities of single pure compounds from very complex mixtures. Cycle times for PrepLCMS analyses are 10 min or less, thereby permitting purifications in excess of 100 compounds per day per PrepLCMS instrument. Preliminary results of this method are presented for the separation, purification and isolation of compound libraries derived from parallel synthetic strategies.

2. Materials and methods

2.1. HPLC instrumentation

Analytical HPLC analyses were performed using a Shimadzu LC10AD binary high-pressure gradient system (Shimadzu, Columbia, MD) controlled through the PE-Sciex (Ontario, Canada) Sample Control software. A Shimadzu SPD-10A dual wavelength detector was used to acquire UV spectra at λ=220 nm and λ=254 nm.

Preparative HPLC analyses were performed using a Shimadzu LC8A binary high-pressure gradient system controlled through the same Sample Control software. No UV detection was recorded for preparative scale analyses.

2.2. Analytical HPLC conditions

Analytical HPLC separations were made using either a Poros R2/H 10 cm×300 μm perfusion column (PerSeptive Biosystems, Framingham, MA) or a 5-μm, 300 Å Monitor 4.6-mm I.D.×5-cm C_{18} column (Column Engineering, Ontario, CA) or alternatively, a 4.6-mm I.D.×5-cm C_{18} YMC column (YMC, Wilmington, NC). For the perfusion column experiments, the column flow was set to 1.0 ml/min. Following an initial hold at 10% Buffer B for 1 min, compounds were separated on the perfusion column using a gradient of 10% to 70% Buffer B in 6 min, with a hold at 70% Buffer B for 2 min. Equilibration time between analyses was 1 min. For the C_{18} column experiments, the column flow was set to 1.2 ml/min. Following an initial hold at 10% Buffer B for 1 min, compounds were separated on the C_{18} column using a gradient of 10% to 61% Buffer B in 4.5 min, and 61% to 91% Buffer B in 2 min. Equilibration time between analyses was 1 min.

2.3. Preparative HPLC conditions

Preparative HPLC separations were achieved using either a Poros R2/H 10-mm I.D.×10-cm perfusion column (courtesy of Jeff Wilson, PerSeptive Biosystems,) or a 5-μm, 300 Å Monitor 21.2-mm I.D.×5-cm C_{18} column (Column Engineering, Ontario, CA). For the perfusion column experiments, the column flow was set to 10 ml/min. The gradient was the same as described for the analytical Poros R2/H analysis. For the C_{18} column experiments, the flow-rate was set to 20 ml/min. The gradient was the same as described for the analytical C_{18} analysis. For large scale purifications, a 30-mm I.D.×7.5-cm Monitor column was used. The gradient was the same as described for the 20 mm I.D. column and the flow-rate was set to 50 ml/min.

2.4. Electrospray ionization mass spectrometry

All analyses were performed using a PE Sciex API100LC or API100 single quadrupole mass spectrometer equipped with an electrospray ionization source. The mass spectrometer was operated at unit resolution up to *m/z* 1200 (10% valley definition). Mass spectra were acquired by scanning from *m/z* 200–1200 in 3.3 s using a dwell time of 1.0 ms and a

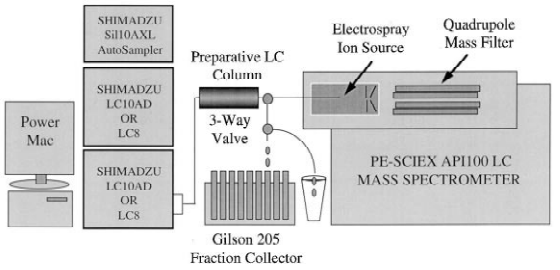


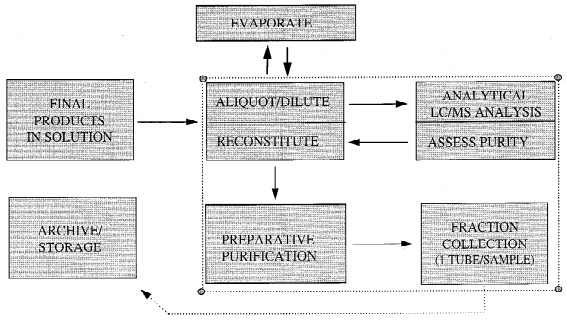
Fig. 1. Schematic representation of the Analytical/PrepLCMS system. For semipreparative HPLC–MS purifications, a high-pressure gradient is formed using Shimadzu LC10AD pumps. For preparative scale purifications, Shimadzu LC8 pumps are used. A valco tee is situated at the outlet of the preparative column. A very small proportion of the preparative column flow is directed into the mass spectrometer ion source (less than 100 μl/min). The remaining flow is diverted to a 3-way switching valve which is positioned directly above a Gilson 204 (or 205) fraction collector. The 3-way switching valve is normally configured to divert to waste and only switches to fraction collect when the mass spectrometer 'triggers' the valve to rotate to fraction collection.

step size of 0.3 Da. Less than 1% of the total column effluent was diverted through a valco tee into the electrospray ionization source housing. The remaining >99% of the column flow was diverted through the same tee into a 3-way valve positioned directly above a Gilson 205 fraction collector (Gilson, Madison, WI), as shown in Fig. 1. Fraction collection was triggered when the reconstructed ion current (RIC) for the $(M+H)^+$ ion reached a threshold level of 1×10^6 cps. Fraction collection termination time was generally set to 30 or 60 s, as governed by the volume of the fraction collector vials (15 ml) and the column flow-rate.

3. Results and discussion

For biological assays, it is important that the compound being screened be of known concentration and purity so that reliable information regarding the structure–activity relationship can be gleaned from the biological data. Although there is debate as to what constitutes an acceptable level of purity for biological screening, correlating biological activity (or inactivity) to compound structure clearly occurs with greater confidence when a compound is at least 85–90% pure. NMR and thin layer chromatography have been used traditionally by the medicinal chemist and the data often give the appearance of purity when in fact, the compound is shown to be impure by other analytical methods, such as HPLC or HPLC–MS. HPLC–MS incorporating UV and/or ELS detection has been considered to be a much more reliable method for assessing pharmaceutical compound purity. Analysis times for traditional RP–HPLC–MS has precluded its utility for characterizing compound libraries generated via automated, parallel synthetic strategies, due to the large numbers of compounds afforded by this technique. Recently, ultra-fast, automated methods for chromatographic separation, characterization and purity assessment of these compound libraries have been implemented.

The automation process that has been incorporated in our laboratory for the analytical characterization and preparative scale purification of compounds produced by parallel synthesis is shown in Scheme 1. Final products of the reaction synthesis (either solution or solid-phase) are aliquoted to a concentration of approximately 0.1–0.5 mg/ml either manually or by using a Gilson 215 liquid handling station controlled through the PE SCIEX data system software. The chemist specifies the acquisition method, autosampler vial position and the expected mass



Scheme 1. Synthetic products, whether derived by solid- or solution-phase parallel synthesis, are aliquoted and diluted for analytical LC–MS purity assessment by aid of a Gilson 215 liquid handling station. While analytical assessment is being carried out, parallel synthesis libraries are evaporated off-line using a speed-vac Savant evaporator/concentrator. Samples found to be greater than 85–90% pure (based on an average of both the UV_{220} and UV_{254} measurements) are transferred to a robotics workstation for sample archiving and plating for biological assay. Samples falling below the purity criterion of 85–90% are reconstituted in DMSO to a concentration of 30–60 mg/ml and are subjected to on-line, automated PrepLCMS purification.

of the desired compound in a Microsoft Excel spreadsheet. The spreadsheet is imported into the data acquisition program where analytical assessment of the compound library is made using a fast gradient as described in the Section 2. Data processing and purity assessment is performed automatically, immediately following data acquisition for each compound. Applescripts permit compound purity assessments to be made automatically (via integration of both the λ_{220} and λ_{254} absorbance measurements) and the results are exported to Microsoft Excel for evaluation.

High resolution chromatographic separations are made possible using short, 4.6-mm I.D.×5-cm columns and fast gradients. A typical data output file for a single compound with at total analysis time of 10 min (or less) is shown in Fig. 2 below. The script plots the reconstructed ion chromatogram (RIC) for the compound of interest to determine its retention time. The script proceeds by integrating the area under the λ_{220} and λ_{254} peaks whose retention time falls within a 0.1 min window of the retention time of the RIC peak (at its peak maximum). The purity of the compound is defined and the chromatogram labeled. For each compound library that is characterized, a purity table is produced in the form of an excel spreadsheet, from which compounds are sorted based on their average purity. Compounds with average purities greater than 85–90% are transferred to a robotics workstation for archiving and dispensing into microtiter plates for screening.

Often, because many of the chemistries employed are neither simple nor high yielding (e.g., multi-step reactions), a purification step is required. Fig. 3 shows the results of analysis of a compound, whose purity falls below the 85–90% threshold level. To facilitate the purification of this (and similarly impure) compounds, and, in order to ensure all bio-

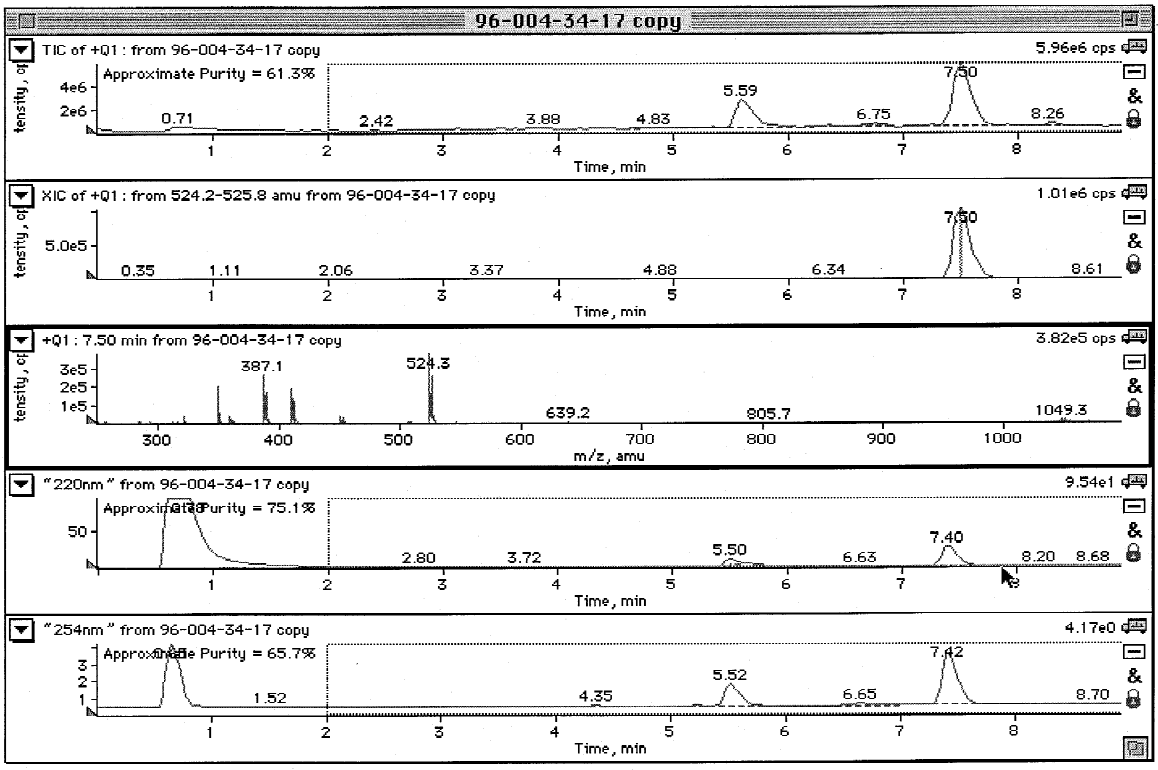


Fig. 2. Flow chart for the fraction collect AppleScript. In addition to the script itself, a small scriptable application was written to control the Gilson fraction collector. The script actually sends messages to this application when the fraction collector needs to be advanced or when the diverter valve needs to be flipped. Currently this application can control a Gilson FC 203, FC 204 or an FC 205.

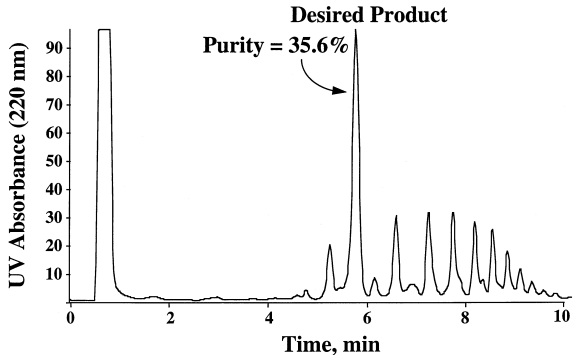
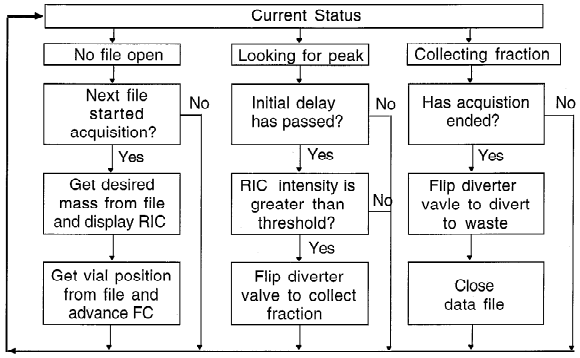


Fig. 3. An example of a parallel synthesis gone awry. The 'desired' compound is found to have a purity of less than 30%, as determined from an average of both the UV_{220} and UV_{254} absorbance measurements.

logical assay data can be appropriately correlated to the compound being screened, we have developed a fully automated, rapid and novel method for the preparative scale purification of compound libraries. This method involves the use of a mass spectrometer to trigger fraction collection, as pictorially represented in Fig. 1. The mass of the desired compound is entered into the same data acquisition program that is used for purity assessment. Applescripts are used to facilitate communication between the 'real-time' mass spectrometer data acquisition and the fraction collector, as delineated in Scheme 2. The 'user' specifies a threshold RIC for initiating fraction collection, a threshold for terminating fraction collection and a collection time, in the event that the desired eluting peak fails to drop below a minimum threshold setting, as shown in Fig. 4.

Shown in Fig. 5A–D are the results of the first automated preparative purification of compound libraries using a mass spectrometer to trigger fraction collection [31]. Fig. 5A shows the UV chromatogram for the analytical LC–MS assessment of a compound derived by parallel solution phase synthesis. The desired product is found to have a purity of ≤40%. Fig. 5B shows the UV chromatogram for a 0.5 mg injection of the same compound onto a semi-preparative (10-mm I.D.) column. The chromatographic separation is nearly identical to a smaller sample loaded onto a capillary column (0.3 mm I.D.). Fig. 5C shows the purification of a 10-mg sample loaded onto the semi-preparative column. The RIC of the



Scheme 2. The script opens each data file as soon as the first scan has been acquired. It then displays a reconstructed ion chromatogram (RIC) for the desired mass. Immediately, the fraction collector advances to the last tube contained within the fraction collector and washes the line (to eliminate the possibility of cross-contamination from one analysis to the next). Following this brief washing step, the fraction collector advances to the specified tube (from the autosampler) and monitors the RIC. When the script detects that the intensity of the RIC is above a specified threshold, it triggers the fraction collector to switch from 'waste' to 'collect' through RS232 communication to the three-way switching valve. This initiates fraction collection. After a fixed length of time (1 min in this case) or when the signal drops below the specified threshold value, the effluent is again directed to waste.

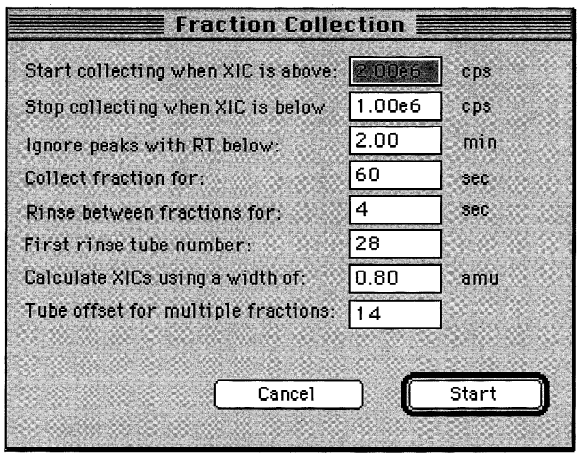


Fig. 4. The threshold for initiating and terminating fraction collection is set to a default setting of 1×10^6 cps unless otherwise specified by the end-user. The fraction collect time is set to the maximum collection volume. For example, if the fraction collector tube has a 15 ml volume and the column flow-rate is 20 ml/min, the collect fraction time is set to a maximum of 45 s, to ensure that no fraction collected will exceed the volume of the fraction collector tube.

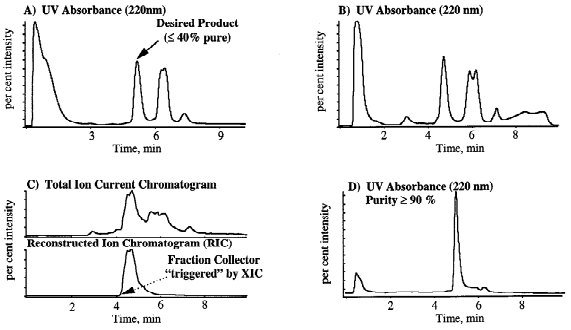


Fig. 5. (A) UV chromatogram of an impure compound separated on a Poros R2H perfusion column (0.3 mm I.D.×10 cm). Purity assessment is determined based on averaging of both l_{220} and l_{254} integrated areas. (B) 10-mg injection of same compound onto the semipreparative LC column. 0.1% of column effluent is diverted to the mass spectrometer, 99.9% of effluent is diverted to a 3-way valve positioned directly above a Gilson 204 fraction collector. Fraction collection is initiated when the RIC for the desired compound reaches a threshold value of 1×10^6 cps. (C) purity assessment following PrepLCMS showing ≥90% purity.

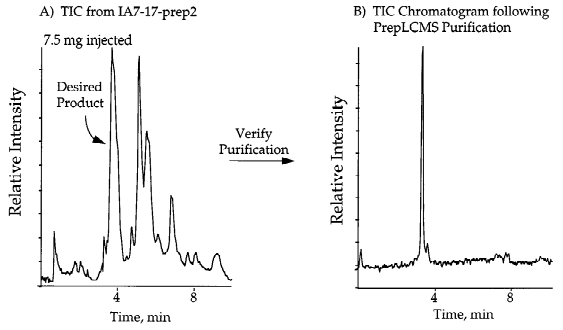


Fig. 6. PrepLCMS purification of a compound derived by parallel synthesis whose initial purity is determined to be <85%. Purification using a Poros R2H 10-mm I.D.×10-cm perfusion column is shown. (A) TIC chromatogram observed from a 7.5-mg sample injection and (B) TIC chromatogram following PrepLCMS, demonstrating the efficient purification of the desired compound.

desired product is monitored in real-time and fraction collection is initiated when the RIC threshold exceeds 1×10^6 cps. Fraction collection in this case was terminated by setting a collection time of 60 s (using an earlier version of the script which did not allow fraction collection to be terminated based on a signal threshold being observed). The resultant purified product was evaporated, reconstituted in DMSO to a concentration of 10 m*M*. Fig. 5D shows the UV chromatogram for the analytical assessment of an aliquot of the purified product. The peak observed at approximately 30 s is the DMSO solvent peak. The purity of the compound, following PrepLCMS purification is greater than 90%. A second example of the performance of the PrepLCMS method for the purification of compound libraries by perfusion chromatography is shown in Fig. 6. Using the identical chromatographic methods and threshold settings, the desired compound, showing an initial purity far below 85%, is, following on-line PrepLCMS, purified to greater than 95%. Table 1 summarizes the results of the PrepLCMS purification of a subset of compounds from an iminodiacetic acid library solution phase parallel synthesis.

During the course of the development of the PrepLCMS method, we found limited utility of semipreparative perfusion chromatography for purification of combinatorial libraries. This was attributed to (a) the poorer column efficiency for small molecule separations on a Poros R2H perfusion column relative to C_{18} reversed-phase columns and (b) relatively low loading capacity for small molecule separations. Improvements in both column resolution and 'purifyable' quantities was made using larger inner diameter reversed-phase C_{18} columns. The results of a 24-mg injection of a compound library component purified by fast PrepLCMS on a 21.2-mm I.D. C_{18} Monitor column is shown in Fig. 7. Table 2 summarizes a batch library purification by PrepLCMS using the same 21.2-mm I.D. C_{18} column. Samples were solubilized in DMSO to a concentration of 60 mg/ml and aliquots corresponding to 24-mg injections were made on-column (unless specified). Thresholds for

Table 1
Automated semipreparative perfusion column LC–MS

File name	Purity (Q/C)	Purity (prep)	Amount purified (mg)
IA7-11	63.1%	≥99%	2.7
IA7-12	49.7%	90.0%	2.4
IA7-13	53.0%	86.0%	2.6
IA7-14	70.5%	≥99%	2.1
IA7-15	78.4%	≥99%	3.6
IA7-16	79.1%	≥99%	3.0
IA7-17	58.9%	≥99%	3.7
IA7-18	80.8%	93.0%	3.8
IA7-19	72.5%	91.0%	1.8
IA7-20	40.0%	85.0%	0.5

Column and conditions: Poros R2/H 10 mm I.D.×10 cm, 10 ml/min, 1% to 71% B in 7 min.

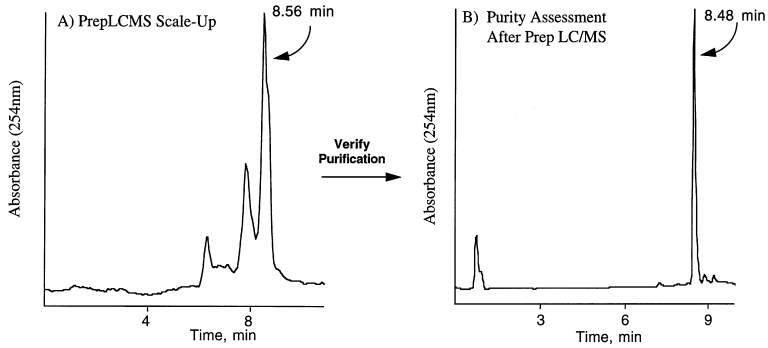


Fig. 7. PrepLCMS purification using a Monitor reversed-phase C_{18} column (21.2 mm I.D.×7 cm). (A) UV chromatogram of a 24 mg-sample analyzed by PrepLCMS shows the desired compound is approximately 50% pure. (B) UV chromatogram of PrepLCMS purified compound, lyophilized and reconstituted in DMSO, showing greater than 90% purity.

initiating and ending fraction collection were set, as well as a collection time were as described earlier. Table 2 shows the results of the batch purification of 20 compounds from a parallel synthesis that failed to meet the 85–90% purity criterion for biological screening. These compounds were batch purified unattended, and overnight by PrepLCMS (total analysis time of 400 min). Of the 20 compounds subjected to on-line, unattended PrepLCMS, 18 samples passed the purity criterion for screening following PrepLCMS. Two of the twenty compounds showed final purities below 85%. These two compounds were minor components within their given mixtures and upon scaling up for preparative purification, a degradation in their chromatographic separations was observed. These two samples were subjected sub-

Table 2
Automated preparative C_{18} column LC–MS

Compound ID	Initial Purity (per cent)	Final Purity (per cent)	Conc (mg/ml)	Amt. Inj. (mg)	Amt. Purified (mg)	Yield (per cent)
ID7-2	83.4	≥99.0	60.0	24.0	18.0	89.9
ID7-3	66.8	≥99.0	60.0	24.0	15.3	95.4
ID7-4	57.7	≥99.0	60.0	24.0	11.0	79.4
ID7-5	29.5	85.0	57.4	23.0	6.0	74.3
ID7-7	43.1	≥99.0	33.2	13.3	6.1	≥100
ID7-8	22.5	≥99.0	17.4	7.0	1.5	95.2
ID7-9	38.2	91.6	60.0	24.0	6.8	68.0
ID7-12	36.1	≥99.0	60.0	24.0	6.1	70.4
ID7-13	21.8	90.5	60.0	24.0	4.2	72.7
ID7-17	29.0	73.0	26.8	10.7	1.5	66.2
ID7-18	47.4	≥99.0	60.0	24.0	5.9	51.9
ID7-19	19.3	75.4	60.0	24.0	6.9	≥100
ID7-20	13.2	≥99.0	60.0	24.0	4.7	≥100
96-004-34-1	63.5	93.3	60.0	24.0	12.6	82.7
96-004-34-2	73.7	≥99.0	60.0	24.0	13.3	75.2
96-004-34-7	70.7	≥99.0	60.0	24.0	16.0	94.3
96-004-34-9	83.2	≥99.0	60.0	24.0	11.4	57.1
96-004-34-14	24.8	86.5	55.0	22.0	2.3	48.7
96-004-34-17	65.6	≥99.0	60.0	24.0	11.5	73.0
96-004-34-19	54.8	87.0	60.0	24.0	6.6	57.7

Column and conditions: Monitor C_{18} 25.4 mm I.D.×7.5 cm, 20 ml/min, 1% to 71% B in 7 min.

sequently to a modified, shallow gradient which permitted their separation and purification to greater than 90% (data not shown). Even so, many of the compounds with initial purities of less than 20%, showed that it was possible to purify multimilligram quantities to greater than 90% by PrepLCMS.

It was found that some compounds showed very high yields (≥90%) following PrepLCMS whereas some of the other compounds showed yields, following purification of approximately 50%. The variations in yield are likely correlated to the on-column behavior of the individual compounds. Chromatographically well-behaved compounds would likely be purified with high yields whereas chromatographically poorly-behaved compounds would likely be purified with lower yields (due to precipitation on-column, partial retention on-column, etc.). In order to evaluate the performance of the PrepLCMS system and to determine that the system was configured to permit high yield compound purifications, replicate injections of a standard compound were made, as shown in Fig. 8. For this standard compound, yields were greater than 90% for an $n=4$ replicate injections, demonstrating that when a compound exhibits 'good' chromatographic peak shape, the PrepLCMS method does not lead to significant sample losses.

In many cases, it is desirable to perform even larger scale purifications, so that the compound is available for multiple in vitro and in vivo assays. We have used larger inner diameter reversed-phase columns operated at a higher flow-rate for this purpose. In addition, this has required the implementation of larger fraction collection beds to accommodate these higher flow-rate columns. Shown in Fig. 9 is the configuration of the PrepLCMS system for large scale (20–50 mg) purifications. The Gilson 204 fraction collector bed holds a total of 56, 50-ml centrifuge tubes. The last tube within the fraction collector bed is reserved for washing of the 3-way valve at the beginning of the analysis. Within the first few seconds of the analysis, the script directs the fraction collector to move to position 56 and to flip the switching valve to collect for a user specified amount of time (typically 3–5 s). Following this initial wash of the tubing just below the 3-way switching valve, the fraction collector moves to the fraction collector position defined by the autosampler

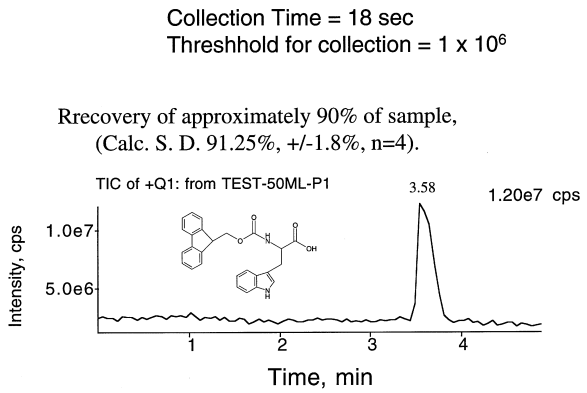


Fig. 8. Replicate 30-mg injections ($n=4$) of a standard iminodiacetic acid library compound onto the PrepLCMS system using a Monitor C_{18} 25.4-mm I.D.×7-cm column. The peak width (10% valley definition) was 18 s. Threshold for fraction collection was set to 1×10^6 cps and collection time was set to 18 s. The purified fraction was collected into a tarred 15-ml conical vial. Following purification, the sample was lyophilized and the vial weighed. The sample recovery for an $n=4$ replicate injection was 91.3%±1.8%.

vial position. Fraction collection is initiated upon a threshold signal being observed for the compound mass of interest. An example of a fast PrepLCMS purification of a single 50-mg sample from a queue of 40 samples purified overnight is shown in Fig. 10.

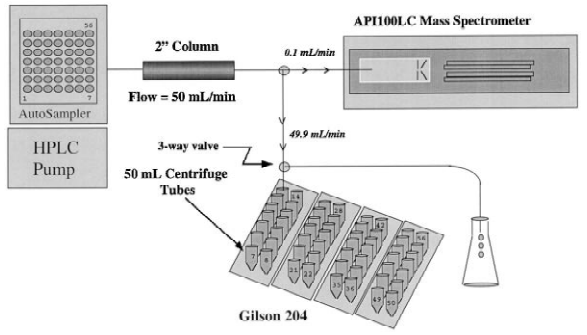


Fig. 9. Configuration of PrepLCMS system for larger scale purifications. A 30-mm I.D.×7.5-cm column, operated at flow-rate of 50 ml/min, is used for rapid multimilligram purifications (50 mg). At 50 ml/min, fractions are collected into 50-ml centrifuge tubes. For this size tube, the Gilson 204 fraction collector bed permits up to 55 automated and unattended purifications to be performed by PrepLCMS. For lower scale purifications, fractions may be collected into 15-ml conical tubes. The Gilson 204 bed permits collection of up to 176 automated unattended purifications.

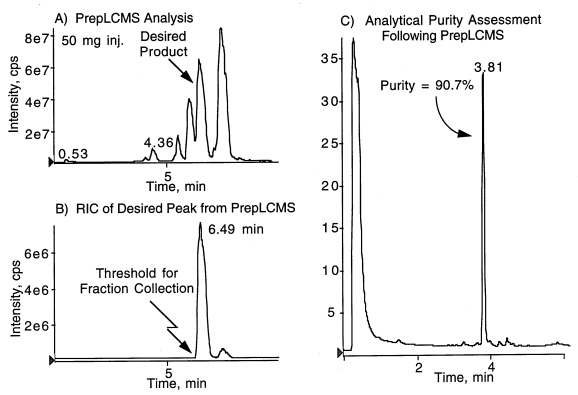


Fig. 10. (A) A total of 50 mg of crude reaction product was injected onto a 30-mm I.D.×7.5-cm C_{18} column. Shown is the TIC chromatogram. The complexity of the crude reaction mixture required a shallower gradient to be used (10% to 40% ACN in 3 min, 40% to 60% ACN in 4 min) for the preparative scale purification. The purity of the desired product was determined to be less than 50%. (B) RIC of the desired product from this 50 mg injection. Fraction collection was initiated when the RIC signal reached a threshold level of 1×10^6 cps. In this instance, fraction collection was terminated upon the signal threshold decreasing to 1×10^6 cps. (C) An aliquot of the purified product was injected onto a 4.6-mm I.D.×5-cm column and analyzed. The purity was determined to be greater than 90%. The purified product was reconstituted in DMSO to a solution concentration of 10 m*M* and plated for biological screening.

It was found that 38 of the 40 samples were suitably pure and isolated in multimilligram quantities for biological screening after PrepLCMS.

We have incorporated both the analytical and preparative LC–MS capabilities recently into a single fully integrated system which is used as a 'walk-up' system by individual chemistry teams. Fig. 11 shows the analytical/preparative HPLC–MS system,

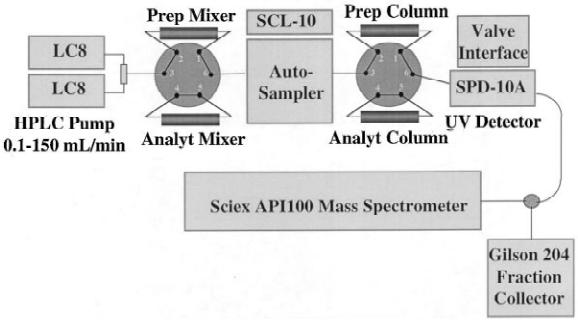


Fig. 11. Automated, 'open-access' analytical/preparative HPLC–MS for assessment of compound libraries.

which has been designed specifically for the chemist to permit unattended purity assessment and purification of compounds derived by parallel synthesis. A single set of preparative HPLC pumps is used for both analytical and preparative HPLC analyses. All of the components of this system are under computer control, including gradient control, switching valves, autosampler, dual wavelength UV detector and fraction collector. The chemist specifies within the acquisition method either ''Analytical LC–MS method or PrepLCMS method''. The switching valves rotate to the appropriate configuration to facilitate either analytical or preparative-scale analyses. The system has been designed for maximum flexibility, permitting semipreparative and large scale preparative purifications while at the same time, permitting lower flow-rate analytical HPLC–MS for purity assessment.

4. Conclusions

Demonstrated within was an automated system for the rapid purity assessment and purification of compound libraries derived by parallel synthesis strategies. Rapid characterization of compound libraries was achieved using steep gradients on short reversed-phase columns. Automated semi-quantitative purity assessment was made by aid of Applescripts for post-data acquisition processing. Automated purification of compound libraries was made possible by incorporating intelligent fraction collection, using a mass spectrometer to trigger fraction collection based on the real-time reconstructed ion current signals. Proper execution of the PrepLCMS method requires that the mass and autosampler position for the desired compound be identified in the sample acquisition software. The reconstructed ion chromatogram for the mass of interest is monitored in real-time as a function of the preparative scale purification. In this manner, only the compound of interest is collected, thus permitting batch purifications to proceed in a fully automated, unattended manner.

The method was demonstrated for both semi-preparative and preparative scale purifications. It was found that the mass spectrometer performs equally well, whether large scale samples (50 mg) or small

scale sample (1–10 mg) are injected on-column. This is because only a small fraction of the column effluent at any given time (ca. 0.1%) is diverted into the mass spectrometer. In general, semipreparative purifications have been required when only initial screening data is required (e.g., determination of percent inhibition). When 'full-blown' IC_{50} measurements are required and/or when compound archiving is needed, typical of many pharmaceutical research programs, preparative scale, multimilligram purifications are performed. Preparative scale PrepLCMS purifications (50–100-mg quantities) have required the use of larger columns (typically 20–30-mm I.D.). To minimize equilibration times and hence, maintain optimal analysis speed, these columns are operated at flow-rates of up to 50 ml/min. Performing 'batch' purifications (50–100 compounds per night) on larger inner diameter columns operated at higher flow-rates necessitates the use of significant amounts of solvent (greater than 20 litres per night) and the production of significant volumes of waste solvents, two issues not to be trivialized.

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