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An automated LC method for the small-scale purification of organic molecules derived from combinatorial libraries

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Abstract

This paper presented the development of an automated HPLC small-scale purification method for single bead compounds derived from combinatorial libraries. The method was found to produce higher and more consistent recoveries of purified compounds as compared to conventional manual HPLC purification. Using the manual method, the average percentage recovery of one synthetic compound was determined to be 24% and the coefficients of variation (C.V.%) of recovery were found to be greater than 38%. Using the automated system, the average percentages recovery of a standard compound at 600 and 1000 μmol l^{-1} were determined to be $72.63 \pm 10.17\%$ and $81.34 \pm 4.39\%$, respectively. This represented an approximate 3-folds increase in percentage recovery compared to that of the manual small-scale purification process. It was also found that the C.V.% of recovery were less than 15% at both concentration levels. The development of this automated method was found to be straightforward. The importance and implications of this study were discussed.

Keywords: High-performance liquid chromatography; Combinatorial libraries; Small-scale purification

1. Introduction

Synthesis and testing of mixtures of compounds in a combinatorial library offer the potential of much greater throughput than the 'one compound, one well' approach. When mixtures of compounds are screened, however, a 'deconvolution' method must be used to determine which molecule(s) in the library is responsible for the activity. One of the strategies is iterative deconvolution via subset organization of mixtures or 'pooling strategies' [1,2]. However, this method involves numerous steps of retesting of subsets of mixtures until a unique compound is determined; this may be time-consuming and tedious. Another strategy is to analyze individual compounds derived from the deconvolution mixture using reversed-phase HPLC with chemilu-

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minescent nitrogen, UV, and mass spectrometric detection [3]. By combining the concentration data with the biological activity data of these samples, compounds may be accurately rank-ordered for potency. The latter strategy is rendered possible by the use of 'one bead, one compound' solid-phase combinatorial synthesis [4–7].

However, other impurities are often present with the intended compound when it is cleaved from the bead. To verify that the intended product is the primary source of biological activity, biological screening of the pure compound is therefore essential. Hence, small-scale purification of single bead compound is often requested when the intended compound exhibits positive biological activity and when verification of this lead compound is desired. Sufficient amount of the lead compound is required for its biological activity verification. This lead compound, however, is normally present in the low microgram range in 5–8 wells (depending on the size of the mixtures) when cleaved from the beads. Therefore, by pooling the contents from these wells prior to purification, small-scale purification also serves to pre-concentrate the lead compound prior to biological activity assay.

In small-scale purification, we collect the desired fractions manually by HPLC for two reasons. Firstly, unlike large-scale purification, there is only one purification run per sample. Secondly, the number of samples per request in small-scale purification is usually small. Although this manual method is simple, we did not fail to notice the high variations in terms of recovery and the occasionally loss of samples. Since the lead compound is valuable and its amount needed for biological screening is critical, the recovery of the desired fraction during small-scale purification has to be sufficiently high (at least more than 35%) and consistent. There is no literature by far that discuss the small-scale purification of solid-phase combinatorial library compounds. In this paper, the high variation in recovery during manual fraction collection was investigated and confirmed. Based on the latter results, an automated HPLC method for small-scale purification of small organic molecules derived from combinatorial libraries was finally developed.

2. Experimental

2.1. Materials

Diphenhydramine (DPH) was purchased from Sigma (St. Louis, MO). All synthetic compounds were synthesized at Chiron Corporation (Emeryville, CA) and provided in solutions in dimethyl sulfoxide (DMSO). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ). Sequencing grade trifluroacetic acid (TFA) was purchased from Pierce Chemical (Rockford, IL).

2.2. HPLC instrumentation

Manual small-scale purification was performed using a Michrom HPLC system (Michrom BioResources, Inc., Auburn, CA). A Hewlett Packard Zorbax Eclipse XDB-C_{18} 5 μm column (50 × 2.1 mm Mac-Mod Inc., Chadds Ford, PA) was used for separation. Solvent A was 0.1% v/v TFA in water and solvent B was 0.1% v/v TFA in acetonitrile. A gradient method of 5–80% solvent B in 5 min was selected for the optimal resolution of peaks. The flow rate used was 1.0 ml min^{-1} throughout the manual collection process. The UV signal was acquired at $\lambda = 214$ and 254 nm. Data from the UV detector were collected on the computer using the EZChrom chromatography data system (Scientific Software, Inc., San Ramon, CA). The fractions of interest were collected manually using 0.005-inches internal diameter peek tubing connected to the UV detector.

Automated small-scale purification was developed using a liquid chromatographic system that comprised a binary gradient semi-micro pump system (model LC-10AD VP), an on-line degasser (model DGU-14A), an auto-injector (model SIL-10AD VP), a fraction collector (model Frac-10A), a UV-visible detector (model SPD-10A VP) and a system controller (model SCL-10A VP) (Shimadzu Co., Columbia, MD). The liquid chromatographic conditions used were similar to those of manual small-scale purification except those fractions of interest were collected automatically using the fraction collector. The automated HPLC system was controlled using the Class VP software (Shimadzu Co.).

Lead verification of the purified compound was performed using a modular, automated HPLC system comprising a LC delivery system, an UV detector, a chemiluminescent nitrogen detector (CLND) and a mass detector. The set-up and operation of this system had been previously reported [3].

2.3. Investigation of manual small-scale purification

A pure synthetic compound, with a retention time of 1.9 min, in DMSO was used in this part of the study. Five microlitres of this solution was added to 10 μl of 50% v/v acetonitrile. Fifteen microlitres of this mixture was injected into the Michrom HPLC system and the fraction of interest was collected as described. The signal of the peak obtained ranged from 0.8 to 1.0 absorbance unit at 214 nm. The peak area was integrated (PA1). The fraction was speed-dried and the residue was reconstituted with 5 μl of DMSO and 10 μl of 50% v/v acetonitrile. The reconstituted sample (15 μl) was re-injected into the HPLC system. The peak area was integrated (PA2). Two analysts, one of them experienced and the other new, performed five replicates for this process respectively. Three replicates were also separately collected and analyzed using the LC/UV/CLND/MS system. The percent recovery was calculated using the following equation:

$$\text{Percent recovery} = (\text{PA2}/\text{PA1}) \times 100$$

The delay time of the Michrom HPLC system was estimated by injecting a colored synthetic compound and observing the difference in time between the appearance of the peak in the UV chromatogram and the elution of the colored fraction into the 0.4 ml sample glass vial. This process was performed in duplicates to determine the average delay time.

2.4. Method development of automated small-scale purification

The needle stroke parameter controls the distance that the sampling needle descends. In this study, needle stroke of 41 mm (default) and 43 mm (maximum descend) were evaluated. Fifty microlitres of DMSO solution containing the purified synthetic compound (Section 2.4) was added to 100 μl of 50% v/v acetonitrile in a 1.5 ml glass vial. Fifteen microlitres each of this mixture was transferred carefully to 0.4 ml sample glass vials and injected into the HPLC system using needle strokes of 41 and 43 mm, respectively. The integrated peak areas from these runs were then compared.

To optimize the delay time, 15 μl of the mixture used for the needle stroke evaluation was injected into the system using each of these delay volumes, 90, 110, 130, 150, and 180 μl, respectively. Triplicate samples were injected using each delay volume. Each eluted peak was collected and freeze dried. The residue was reconstituted with 5 μl of DMSO and 10 μl of 50% v/v acetonitrile. The reconstituted sample (15 μl) was re-injected into the HPLC system. The percent recovery for each run was determined as described in Section 2.3.

After the needle stroke of the autosampler and the delay volume of the fraction collector had been optimized, the automated small-scale purification system was tested for its recovery and reproducibility. One hundred and eighty microlitres each of 600 and 1000 $\mu mol\,l^{-1}$ solutions of a purified Chiron compound (99% pure, molecular weight 565) was prepared in DMSO and 50% acetonitrile (1:2). Ten microlitres of these standard solutions (600 and 1000 $\mu mol\,l^{-1}$) was injected in triplicates into the HPLC system using the optimized conditions. The fractions collected were speed-dried and reconstituted with 10 μl of DMSO. One microlitre each of these reconstituted samples was added to 14 μl of 50% methanol (0.1% TFA) in a sample vial. One microlitre each of the standard solutions (600 and 1000 $\mu mol\,l^{-1}$) was also similarly prepared in triplicates. These solutions, prepared from both pre- and post-purified standards, were then analyzed using the LC/UV/CLND/MS method (10 μl each was injected into the system). A calibration curve was generated by injecting duplicate standards (DPH) at 250, 500, 1000, 2000 and 4000 pmol of nitrogen.

Based on the peak area in the nitrogen chromatogram, concentration of the each sample was calculated ($\mu mol\,l^{-1}$). The recoveries of the compound at 600 and 1000 $\mu mol\,l^{-1}$ were then determined based on the concentrations of both pre- and post-purified standards.

2.5. Small-scale purification of two single bead compounds

Two single bead compounds, C496 and C618 (molecular weights 496 and 618), were purified using the automated small-scale purification system. Briefly, DMSO solutions containing C496 and C618 were pooled from 3 and 5 wells, respectively (96 well format, each well containing one single bead compound). These pooled solutions were speed-dried in a 0.4 ml glass vial. Each residue was reconstituted with 5 μl of DMSO and 10 μl of 50% v/v acetonitrile. The samples (15 μl each) were injected using the autosampler and the fractions were collected under the optimized conditions. DPH was used as a standard to validate the automated purification system. Stock solution of DPH at 100 $mmol\,l^{-1}$ in DMSO was accurately prepared. The solution was diluted serially in DMSO to produce a working solution at a concentration of 600 $\mu mol\,l^{-1}$. Fifteen microlitres of the working solution was transferred to a 0.4 ml glass vial, the content was injected and the fraction was collected. This latter procedure was performed in duplicates. All the fractions collected were speed-dried and reconstituted with 10 μl of DMSO. One microlitre each of these reconstituted samples was added to 14 μl of 50% methanol (0.1% TFA) in a sample vial. Ten microlitres each of these final mixtures were injected into the LC/UV/CLND/MS system. A calibration curve was generated by injecting duplicate standards (DPH) at 250, 500, 1000, 2000 and 4000 pmol of nitrogen. Based on the peak area in the nitrogen chromatogram, concentrations of the samples were calculated ($\mu mol\,l^{-1}$). The recoveries of DPH standards were also determined. Both the stock and working solutions of DPH were stored in the dark at -25 °C when not in used.

3. Results and discussion

3.1. Manual small-scale purification

The results in this part of the study are presented in Table 1. The average percentage recovery of the synthetic compound was determined to be 24% for both the experienced and new analysts. This percentage recovery was relatively low and the amount recovered may sometimes be insufficient for further biological studies. It was also clear from the coefficients of variation (C.V.%) that the disparities of the percentages of recovery were high. Percentages of coefficients of variation were 59 and 40% high for the experienced and new analysts, respectively. These high coefficients of variation were also observed in samples analyzed by the LC/UV/CLND/MS system where the average peak area at 214 nm was found to be $553\,241 \pm 303\,558$ ($n = 3$; C.V.% = 55%).

Recently, rapid HPLC methods have been introduced for the analytical characterization of compound libraries [8]. 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. In our laboratory, we adopted these fast HPLC methods for the small-scale purification of single bead

Table 1
Recovery profile of a pure synthetic compound using manual small-scale purification

Replicate	Percentage recovery based on peak area	
	Experienced analyst	New analyst
1	12.26	31.16
2	17.77	26.38
3	44.65	29.02
4	21.35	10.12
5	24.41	ND[a]
Mean	24.09	24.17
S.D.	14.26	9.57
C.V.%	59.19	39.58

[a] ND: not determined, pump leaked during run.

compound so as to increase the throughput of this process. Consequently, the peak observed in the UV chromatogram was generally sharp with an estimated width at half height of 0.05 min. In other words, a particular peak might elute out of the system rapidly in a few seconds (3–6 s). Using a colored synthetic compound, we determined the average delay time of the Michrom HPLC system to be 2.5 s ($n = 2$). This relatively short delay time coupled to the fast elution of peak might have rendered manual collection inconsistent and difficult.

From these results, we confirmed that manual small-scale purification of single bead compound was inconsistent in recovery. Since the experienced analyst produced similar results as the new analyst, it was clear that this phenomenon was not exclusive to the latter. This implied that the small-scale purification process might have to be improved to achieve consistently high recovery of the single bead compounds.

3.2. Automated small-scale purification

Since the sample was injected automatically instead of manually using a syringe, optimization of the needle stroke of the autosampler was performed. For the needle stroke experiment, it was found that the average losses of compound were 23 and 14% when the samples were injected using needle stroke levels of 41 and 43 mm, respectively. Hence, there was an approximately 10% reduction in sample loss when the needle was lowered further by a distance of 2 mm. Since the furthest distance that the needle could plunge into the sample vial is 43 mm, we accepted this distance as the optimized needle stroke value for our method.

We attempted to optimize the delay time of the automated small-scale purification system using a colored compound, coumarin. Fifteen microlitres of this compound dissolved in water at 1 mg ml^{-1} was injected and its elution in the fraction collector was observed using a multi-band UV lamp. Although this method was used successfully for our large-scale purification systems, it was found not suitable in this case where the tubing leading to the fraction tubes was not clearly visible. Based on these findings, we subsequently optimized the delay time by varying the delay volume. The results of the delay volume experiment were presented in Fig. 1. As shown in Fig. 1, the percentage recovery reached a maximum at a delay volume of 110 μl. It was found that using delay volumes greater and lesser than 110 μl led to unanimous reduction in percentage recovery. This trend was however expected since collecting the fraction prematurely or belatedly could potentially result in a loss in recovery. For a flow rate of 1 ml min^{-1}, the default delay volume of the fraction collector is 130 μl. As shown in Fig. 1, the percentage recovery at this default value was approximately 10% lower than that at 110 μl. Hence, it was clear from our results that calibrating the delay volume or delay time of the fraction collector was paramount in the method development of a purification system. The delay volume was set at the optimum value of 110 μl for the subsequent experiments.

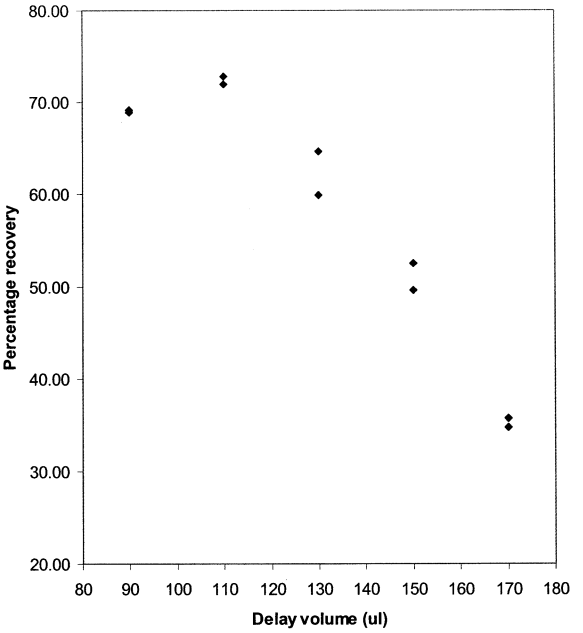


Fig. 1. Plot of percentages recovery against delay volumes of the automated small-scale purification system. Experimental conditions as discussed in Section 2.4.

The recovery and reproducibility of the small-scale purification process were validated using the LC/UV/CLND/MS system. The calibration curve

Table 2
Recovery and reproducibility data of the purification of a pure Chiron compound using the automated small-scale purification method

Levels (μmol l^{-1})	$n=3$	Average concentration[a] (μmol l^{-1})		Average percentage recovery
		Standard (direct injection)	Standard (after purification)	
600		637.81	463.27	72.63
	S.D.	102.43	64.84	10.17
	C.V.%	16.06	14.00	14.00
1000		843.45	686.09	81.34
	S.D.	105.28	36.99	4.39
	C.V.%	12.48	5.39	5.40

[a] The average concentration was calculated using the calibration curve and the nitrogen peak area of each peak.

generated in this part of the study was $y = (42.12)x^{(1.1266)}$ (power curve fit where 'y' was the pmol nitrogen value and 'x' was the nitrogen peak area; r^2 was 0.9998). The curve was generated using 5 concentration points (250, 500, 1000, 2000, and 4000 pmol nitrogen) and two replicates at each concentration (C.V.% at each concentration point were 1.78, 9.74, 1.26, 3.43 and 6.91, respectively). The results of this part of the study were presented in Table 2. The average percentages recovery of the compound at 600 and 1000 μmol l^{-1} were determined to be $72.63 \pm 10.17\%$ and $81.34 \pm 4.39\%$, respectively. This represented an approximate 3-folds increase in percentage recovery compared to that of the manual small-scale purification process (Table 1). It was also found that the C.V.% of recovery were less than 15% at both concentration levels. Compared to the high C.V.% of recovery observed during manual purification ($>38\%$), it was clear that the variations of recovery using the automated small-scale purification method were lower and therefore more acceptable.

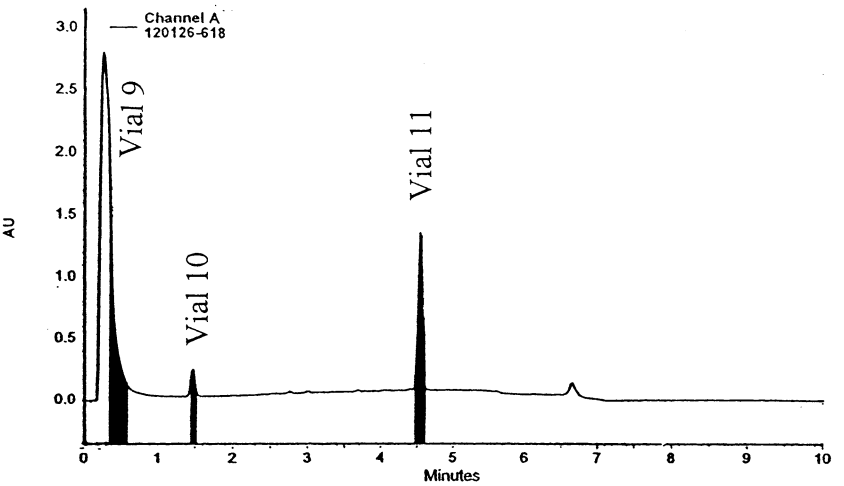
3.3. Small-scale purification of two single-bead compounds

To validate that the automated small-scale purification method was suitable for its final purpose, two single bead compounds from Chiron Corporation were purified using this method. The results showed that the final concentrations of C496 and C618 (molecular weights 496 and 618) in 10 μl of DMSO were 51.44 and 146.90 μmol l^{-1}, respectively. Based on the estimated initial concentrations of these compounds in the wells and the volume of the pooled samples, the percentages of recovery of C496 and C618 were estimated to be 37.5 and 40.38%, respectively. Since there might be loss of samples during pooling, these values were relatively conservative and the actual recoveries could be higher. Nonetheless, these recovery values were still higher than those observed during manual purification. The latter recoveries had been found to vary from 10–20% (data not presented). Hence, there was an observable increase in the recovery of these single bead compounds. As an example, Fig. 2 showed the fraction collection UV chromatogram, based peak chromatogram and full scan mass spectrum of C618. As shown in the fraction collection UV chromatogram, the collection vials and retention times of the major peaks were clearly reported by the Class VP software. This feature would be particularly useful when handling a large batch of samples since the vial of interest could be easily identified from the report and the sample tray. From the base peak chromatogram and the full

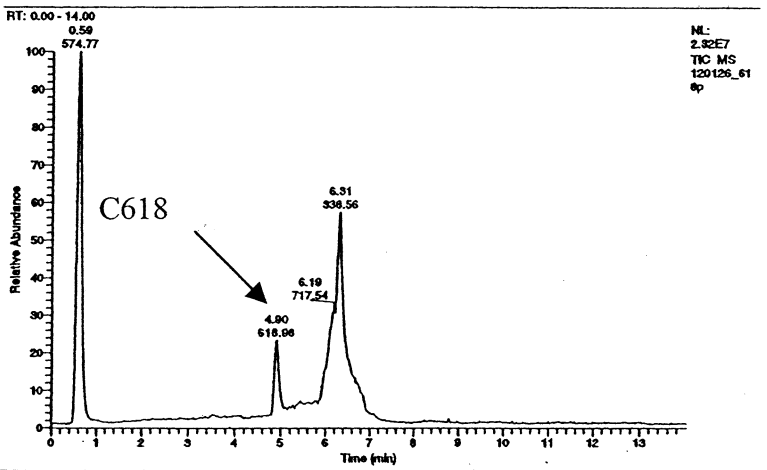
Fig. 2. Purification and characterization of a single bead compound, C618, derived from a combinatorial library. (A) Fraction collection UV chromatogram acquired using the Shimadzu HPLC system. (B) Total ion chromatogram and full scan mass spectrum acquired using the LC/UV/CLND/MS system.

(A)

Fraction Number	Vial Number	Start Time (min.)	Stop Time (min.)
1	9	0.36	0.58
2	10	1.46	1.52
3	11	4.49	4.61



(B)



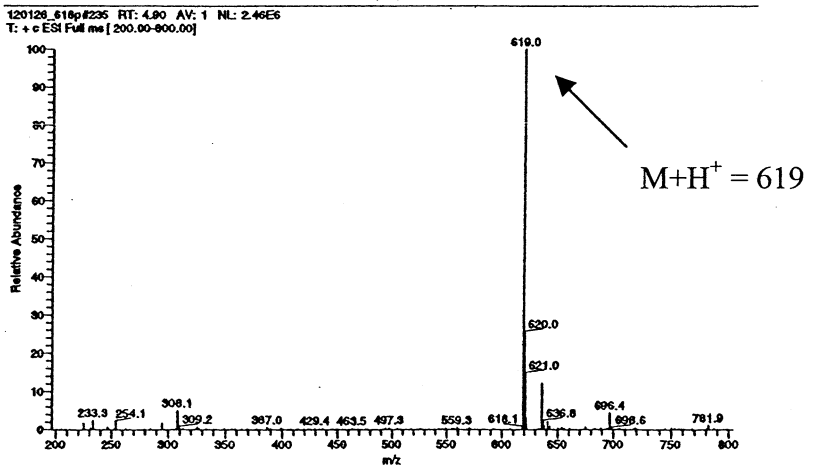


Fig. 2.

scan mass spectrum, it was observed that there was only one major base peak at retention time of 4.90 min; the m/z of the molecular ion $(M+H)^+$ was 619. The 'huge cluster of peaks' observed at 6.29 min was due to the sudden change in the gradient elution of the mobile phases and this phenomenon was observed for all analysis performed by this system. From these results, we therefore verified the concentration, identity and purity of the single bead compound. All these results were essential for the subsequent biological studies.

In this part of the study, two 15 μl aliquots of a working solution of DPH at 600 μmol l^{-1} were also similarly purified. DPH was chosen because it contains a nitrogen atom and could be easily quantified using the LC/UV/CLND/MS system. The aim of purifying the DPH standard was to design a standard protocol for the cross validation of the automated small-scale purification system. The average final concentration of DPH in 10 μl of DMSO was determined to be 452.53 ± 7.46 μmol l^{-1} ($n = 2$; C.V.% = 1.65). Based on the initial concentration and volume of the DPH working solution injected into the system (600 μmol l^{-1} and 15 μl), the estimated recovery of the standard was estimated to be 50.28 ± 0.83 (C.V.% = 1.65). From these results, it was observed again that the consistency of the recovery was better here than the manual small-scale purification process. Further purification confirmed that the high consistency in compound recovery was achievable even when a new analyst operated the system. The procedures of purifying DPH working solution and these accompanying data could be used as part of the standard protocol for cross-validating the automated small-scale purification system. A standard protocol had actually been prepared in our laboratory for this purpose (not presented).

4. Conclusion

The development of an automated small-scale purification method was demonstrated in this study. It was found from the results that the recoveries of single bead compounds using the automated method were higher and consistent when compared to its manual counterpart. This method expedited the purification process since all the steps from injection to collection and reporting were integrated and automated. Similar automated small-scale purification methods could be developed for this type of drug discovery application using other LC systems. We believe that this automated small-scale purification method would contribute significantly to the success of lead compound verification.

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