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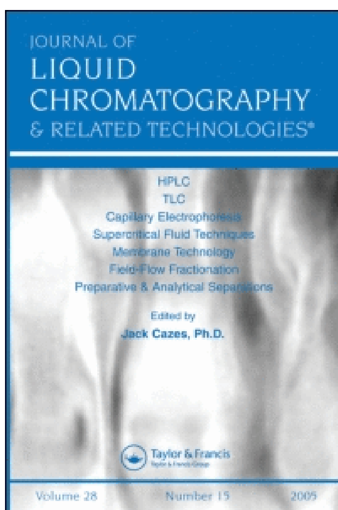

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Streamlined Approach to the Crude Compound Purification to Assay Process

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Streamlined Approach to the Crude Compound Purification to Assay Process

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Abstract: Purification of medicinal chemistry compounds is often the slow step in the drug-discovery process, particularly for compounds generated through parallel synthesis. To address this bottleneck we have developed a fully automated purification platform using a preparative-LC/MS with a customized compound analysis requestor and a data tracking-handling program. The six workstations that comprise the platform are an 8-channel MUX, a mass-directed Prep-LC/MS, a liquid handling system, a balance automator, an evaporation system, and a central PC that runs the core program. The first step involved in the library purification, following the electronic submission of the dry crude synthetic material submitted in a barcoded plate, consists of automatic dissolution of the material and transfer of an aliquot for pre-purification QC. Subsequent steps involve generation of a sequence list consisting of the methods for the analysis and processing, automatic generation of a sequence of the confirmed wells for LC/MS purification, evaporation, fraction dissolution and pooling, automatic weighing, and calculation of the amount needed to make a specified concentration. The final step involves compound distribution into a solubilization tube for the biochemical assay and plates for post-purification QC by LC/MS and NMR. Functions incorporated into the core program were written in CSharp (C#) and Microsoft.NET to create a sample sequence list, annotate wells, track

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purification status, and to enable on-line retrieval of pre- and post-purification data. This platform enables a complete informatics solution from crude compound in a plate to purified compound ready for storage and assay. The processes and results for compounds purified at the multi-milligram level by automated Prep-LC/MS will be described.

Keywords: Streamlined approach, Purification, Process

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INTRODUCTION

The design, synthesis, and characterization of libraries with structural diversity are the key first steps in a pharmaceutical company's discovery process. The need to expedite lead compounds for safety assessment and, ultimately to the market, is crucial in the very competitive pharmaceutical market. Approximately a decade ago, the field of automated high-throughput parallel synthesis emerged[1–3] in order to fill the void where compounds prepared by traditional methods could not fulfill the demand from the capability of high throughput screening. As the number of compounds assayed increased, so did the time and effort required to isolate and characterize active compounds. This situation was further aggravated by the fact that mixtures of compounds can sometimes give false positives or negatives, as well as less reliable structure activity relationship (SAR) data. To address these issues, and develop meaningful SAR data that can be used to guide lead optimization, compound characterization relative to purity and quantity is critical for early discovery processes.

Kibbey et al. first reported, in 1997, the development of an automated system for the purification of combinatorial libraries by preparative HPLC.[4] A two-step approach was reported in which the first involved conducting pre-purification LC/MS to confirm compound compatibility with chromatographic conditions. The second step involved using those results to guide the purification by HPLC with UV triggered collection. Several papers from Kassel et al. followed, where they reported the development of the first mass spectrometry-based purification system using a MS to signal fraction collection.[5–7]

Since the introduction of this new automated purification technology, a substantial amount of time has been devoted to the design, development, and optimization, in order to keep pace with the output of parallel synthesis.[8–16] One such noteworthy advancement by Kassel et al. is the incorporation of a multiplexed MS with multiple columns in order to increase throughput.[8,9] Kyranos et al. presented other technology that has been developed to address the management of different numbers of fractions per compound. The Kyranos work demonstrated one-to-one mapping of fractions to compound mapping which is achieved by enforcing the collection of one fraction per compound injected.[16]

The development of an integrated solution to streamline the purification process to compound registration has been reported by Kassel et al.[9] It was stated that the process steps that included the use of a dual-column LC/MS purification and postpurification analysis did decrease linearly as a function of the number of HPLC columns employed but it was not possible to decrease the total purification process time as a result of this. We have developed and streamlined a complete purification process to decrease the time it takes from the receipt of the crude compound to biochemical screening.

A complete integrated platform and informatics solution, from crude compound in a plate to purified compound ready for biochemical assay and storage, have been developed in our lab. There are four main goals of our platform and informatics solution. First, to improve the quality of compounds going into assays and the compound repository, and to significantly shorten the time it takes to get purified compounds into screening. Second, by using informatics tracking through barcode scanning the compound handling errors can be minimized. A third goal is to have the ability to automatically choose purification parameters and generate a purification sequence list based on the analytical data. Fourth, by integrating all workstations for the purification process, the progress and data tracking can be optimized.

In this paper, the development of a streamlined process and implementation of an integrated high throughput purification platform (HTPP) program that include the functions of the program written in CSharp (C#) and Microsoft.NET to extract, create and disseminate information from the pre-purification to the post-purification steps is described. In addition, a custom-built web-based electronic compound analysis/purification request program using Java, referred to as Analytical Requestor is defined. This application is used to identify, track, and route specific compounds to the analytical chemists, based on their respective functions.

Furthermore, the efficient post-purification sampling handling, namely, the process for routine plate reformatting and the generation of QC plates for purity assessment by LC/MS and NMR is reported. The time required for each step, from the receipt of crude compounds for purification to the generation of pure compounds in vials ready for registration and generation of screening plates for IC50 assay and storage, is also reported.

EXPERIMENTAL

Materials

All compounds used for the recovery and evaluations of the processes were purchased from Sigma-Aldrich (Milwaukee, WI). Compound libraries generated from parallel synthesis by our internal chemistry group were used to assess the robustness of the process. All solvents used were of HPLC

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grade. Acetonitrile (MeCN), methanol (MeOH), dichloromethane (DCM), formic acid (FA), and dimethylsulfoxide (DMSO) were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Water (H_2O) and isopropanol (IPA) were obtained from Burdick and Jackson (Muskegon, MI). Trifluoroacetic acid (TFA) was obtained from Aldrich Chemicals (Milwaukee, WI). YMC ODS AM 5 μm and Gemini C18, 10 μm were purchased from Waters Corporation (Milford, MA) and Phenomenex (Torrance, CA), respectively. The barcoded 24-well and 96-well plates were purchased from Thompson Instruments Company (Oceanside, CA). Fractions were collected into a BLUE MAX™ 50 mL polypropylene conical tubes (Beckson Dickinson Co., Franklin Lakes, NJ). Barcoded Haystack bottles used for compound storage and assay vials for screening were purchased from The Automation Partnership (Royston, UK). The 1000 μL, liquid sensing, non-sterile tips used for liquid and sample transfer are MBP BioRobotix™ (Molecular Bioproducts, San Diego, CA).

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General Instrumentation

The purification platform in our lab includes six workstations that consist of an 8-channel MUX controlled by Masslynx 4.0 software with Openlynx (Waters Corporation), a preparative (pLC/MS) controlled by Masslynx software with Fractionlynx, a Gemini RSP 100 liquid handling system controlled by a version 4.0 software (Tecan Group, Ltd., Zurich, Switzerland), a balance automator controlled by a 4.08 version of the software (Mettler Toledo Bohdan, Vernon Hills, IL), an HT-12 evaporation system (Genevac, Ipswich, England), and a PC that runs the program dedicated to tracking compounds as they move through the process and generate reports.

Analytical LC/MS Instrumentation

All mass determinations were performed on an 8-channel MUX system that has been previously reported by Semin et al.[17] Mass spectra were acquired in the positive ion mode, scanning over the mass range of 150-1,000 Da in 0.15 s.

The analyses were performed using 8 YMC ODS-AM columns (Waters Corp.) with 5 μm particles, 120 Å pores, and column dimensions of 150×2.1 mm i.d. Buffer A was 0.1% TFA in H_2O, and buffer B was 0.1% TFA in MeCN. The total flow rate was set at 3.2 mL/min (i.e., 0.4 mL/min per column). Following an initial hold at 5% for 0.2 min, samples were analyzed on the columns using a gradient of 5%–100% of buffer B in 10 min, hold at 100% buffer B for 2 min, and then returned to initial conditions at 12.5 min.

Preparative LC/MS Instrumentation

The autopurification system (Waters Corporation) consisted of a 2767 one bed injection-collection Sample Manager, a 2525 binary LC pump, a 2487 dual wavelength UV/Vis detector, a ZQ single-quadrupole MS equipped with a Z-spray electrospray interface, a 600F quaternary pump for the make up flow, a UV fraction manager and a 1:1000 ACURATE passive flow splitter from LC Packings (Sunnyvale, CA). The splitter diverts the majority of the column effluent to the 13 mL volume of the UV fraction manager and then to the fraction collector. The remainder of the flow is mixed with the make up solvent of 0.1% TFA in MeOH at 0.75 mL/min and this is, in turn, split between the UV detector set at 254-nm and the MS. The triggering was performed in the positive ion mode, scanning over the mass range of 150–850 Da in 1 s.

The separation was performed using a Gemini column with 10 μm particles, 110 Å pores, and column dimensions of 150 × 30 mm i.d. Buffer A was 0.1% TFA in H_2O, and buffer B was 0.1% TFA in MeCN. The flow rate was set at 45 mL/min. Following an initial hold at 10% for 0.5 min, compounds were purified using a gradient of 10%-100% of buffer B in 14 min, hold at 100% buffer B for 0.5 min, and then returned to initial conditions at 15 min.

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General Operational Workflow and Informatics

Figure 1 illustrates our high-level workflow from compound submission to purified compound in assay and storage formats. Shown in Figure 2 is the informatics workflow driving the high-level workflow in Figure 1. Upon the request and the receipt of the crude plate for purification, the first step is to create a new batch by uploading the relevant information into the HTPP database. This step is followed by dissolution of the compounds in 2 mL MeOH using the Gemini RSP 100 liquid handler and shaking for 5 minutes. If the entire crude batch did not dissolve, as visually observed, then the plate is transferred to a VorTemp™ shaking incubator for an additional simultaneous heating to 40°C and mixing for 5 minutes. Subsequent dissolution steps are taken for compounds that did not dissolve after these initial attempts (e.g., addition of DCM or DMSO). Aliquots of compounds that dissolved are transferred into a barcoded 96-well plate and diluted with MeOH. A sample list for the MUX is generated automatically or custom built with annotations to reflect compounds that did not dissolve. Upon the completion of the analysis and data processing, an automatic report is generated with annotations to either purify compounds that may have failed due to any instrument or compound related reason. At the completion of the purification, fractions are evaporated using the evaporator, and the fractions that are collected in multiple tubes are dissolved in DCM and collapsed into one tube. The fractions are once again evaporated, reformatted into storage vials,

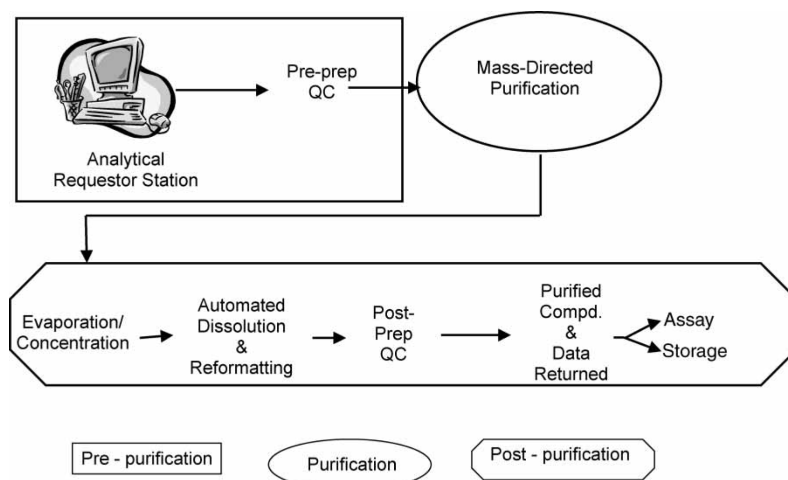

Figure 1. Streamlined operations process for the mass-directed purification platform.

evaporated, and weighed. The compounds are re-dissolved and aliquots are transferred into two barcoded 96-well plates and assay vials. One of the plates is used to determine the post-purification LC/MS and the second plate is used for NMR determination.

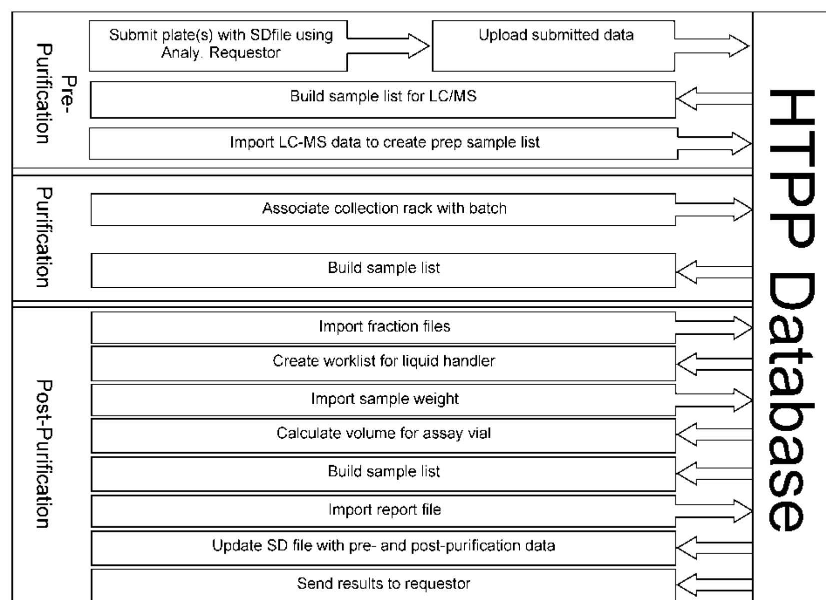

Figure 2. Streamlined informatics process of the mass-directed purification.

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Validation and Case Study

In order to test our processes, perform approximate recovery studies, and implement this platform into our workflow, we used 18 commercially available compounds and 6 plates, each containing 24 Amgen compounds. The weighing of the commercial compounds into the barcoded 24-well plate and intentionally skipping wells, was used to assess the input and output of the process. One function of the HTPP program is to consolidate the positions left empty on the vial rack from compounds that are skipped or not processed with vials containing compounds (e.g., skipped compound vial in original position A1 will be replaced with processed compound vial in position B2, etc.). Compounds submitted in 24-well plates are transferred into 96-well plates in order to efficiently use the 8-channel MUX. For example, the first four rows from the 24-well plate are transferred into the first four rows of the 96-well plate (columns A-D). The next four rows of compounds in the 24-well plate are transferred to the last four rows of the 96-well plate, i.e., compound in the A1 position of the 24 well plate is transferred to the A1 of the 96-well. Compound in the A2 position of the 24-well plate is transferred to the E1 position of the 96-well plate. The liquid handler was used to add MeOH to each well to get an approximate concentration of about 50 mg/mL per well. In order to obtain complete dissolution of all compounds, the plate was transferred to a VorTemp™ shaking incubator for simultaneous heating to 40°C and mixing for 5 minutes. For the pre-purification purity determination, 20 μL from each well were transferred to a barcoded 96-well plate, diluted to 1 mL with MeOH, and 5 μL was subsequently injected onto the analytical LC/MS. Upon completion of the analysis, data processing, and the creation of the purification sequence, 2 mL of the commercial compounds were injected onto the pLC/MS. The fractions were evaporated and reformatted into assay and storage vials. Recoveries were calculated based on the dried weights of the purified compounds and adjusted for pre- and post-purification purities.

RESULTS AND DISCUSSION

Pre-Purification

The purification workflow begins, as shown in Figure 1, with a request for purification generated by a medicinal chemist using the Analytical Requestor. An example of the request page with the input is shown in Figure 3. The first step in the HTPP program is the creation of a new batch, where the data and the SDfile from the Analytical Requestor are extracted and uploaded into the database, as shown in Figure 4. Analytical Requestor is a custom-built web application that allows Amgen scientists to request compound analysis and purification. The application tracks requests,

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Figure 3. INPUT to mass-directed purification platform using the web-based analytical requestor.

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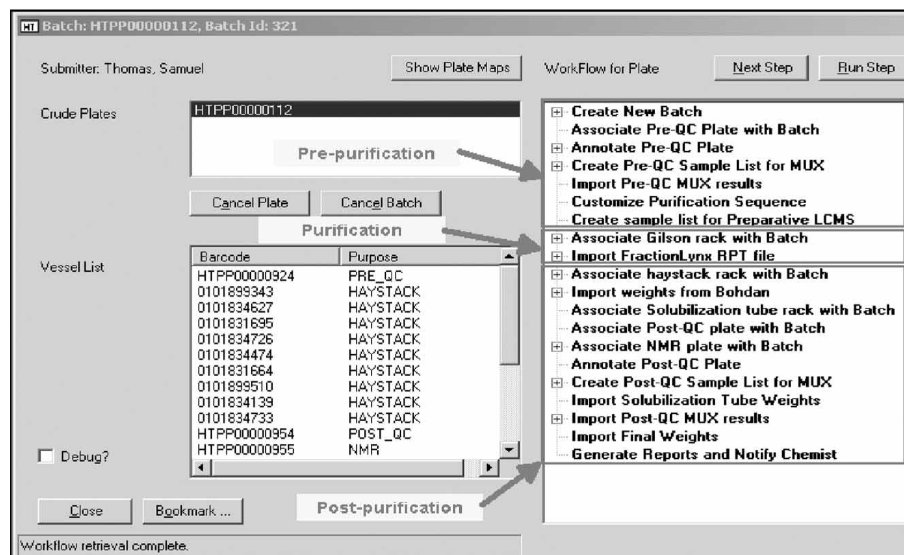

Figure 4. Workflow of the mass-directed purification program written in CSharp (C#) and Microsoft.NET.

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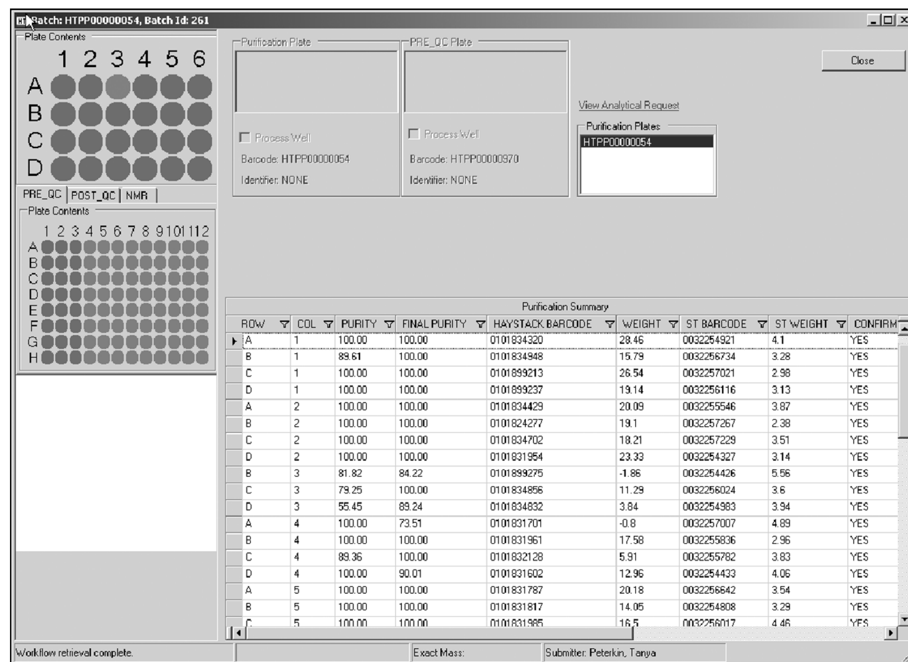

ROW	COL	PURITY	FINAL PURITY	HAYSTACK BARCODE	WEIGHT	ST BARCODE	ST WEIGHT	CONFIRM
A	1	100.00	100.00	0101834320	28.46	0032254921	4.1	YES
B	1	89.61	100.00	0101834948	15.79	0032256734	3.28	YES
C	1	100.00	100.00	0101899213	26.54	0032257021	2.98	YES
D	1	100.00	100.00	0101899237	19.14	0032256116	3.13	YES
A	2	100.00	100.00	0101834429	20.09	0032255546	3.87	YES
B	2	100.00	100.00	0101824277	19.1	0032257267	2.38	YES
C	2	100.00	100.00	0101834702	18.21	0032257229	3.51	YES
D	2	100.00	100.00	0101831954	23.33	0032254327	3.14	YES
B	3	81.82	84.22	0101899275	-1.86	0032254426	5.56	YES
C	3	79.25	100.00	0101834856	11.29	0032256024	3.6	YES
D	3	55.45	89.24	0101834832	3.84	0032254983	3.94	YES
A	4	100.00	73.51	0101831701	-0.8	0032257007	4.89	YES
B	4	100.00	100.00	0101831961	17.58	0032255836	2.96	YES
C	4	89.36	100.00	0101832128	5.91	0032255782	3.83	YES
D	4	100.00	90.01	0101831602	12.96	0032254433	4.06	YES
A	5	100.00	100.00	0101831787	20.18	0032256642	3.54	YES
B	5	100.00	100.00	0101831817	14.05	0032254808	3.29	YES
C	5	100.00	100.00	0101831985	16.5	0032256017	4.46	YES

Figure 5. A typical result screenshot showing all the data generated from the processes.

permits Analytical Chemists to attach results as documents, and automatically sends results to requestors through e-mail. The application was written in Java, J2EE (Java 2 Platform, Enterprise Edition) and uses an Oracle database.

Next, is the association of the barcode, which is scanned into the database, on the 96-well plate to be used for the preparation of the compounds for the pre-purification LC/MS. Upon the completion of the solubilization of the crude compounds, the aloiquots are transferred and diluted. This is followed by the generation of the compound list for the purity and mass confirmation on the MUX platform. Upon the completion of the analysis and processing of the results, the report file is uploaded into a designated network folder and the HTPP program is used to import the results into the database. A customized purification sample list is created; the customization allows compounds that either failed or passed pre-purification for any reason to be processed (Figure 5). Typical reasons for failure are that aliquots are either too dilute, crash out, or mass specified was not found. Aliquots that may be too dilute can still be processed by manually selecting the process option in the customized purification step. This option allows for compounds that failed to be automatically included in the purification sample list.

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Purification

This stage begins with the scanning of the barcode on the first collection rack into the HTPP program and then the importation of the sample list (worksheet) into the purification system under the control of Masslynx software with Fractionlynx (Figure 4). After the completion of the purification and data processing, the Fractionlynx report file (.rpt file) is uploaded into the designated folder on the network and, in-turn, is extracted into the database.

Typical collection is one fraction for each compound but, in some cases, two or more fractions containing compounds of the same masses are collected, due to a variety of reasons. One, compound contained two or more isomers. Two, larger fractions due to a higher yield than expected from the synthesis. Three, collection of a second, third, and, sometimes, fourth mass (e.g., starting material, interesting impurities) are requested. At this time, a proper solution to address this issue has not been established and these fractions are pulled and processed manually.

Post-Purification

The importation of the Fractionlynx report and the association of barcode on the vial rack with the batch into the database is the first step in this stage. Consequently is the combining of multiple fractions, evaporation, transfer of compounds into the storage vials, and the weighing, the compound weights are uploaded into a designated network folder and the

HTPP program is used to import the weights into the database. The program calculates the amount of compound to be transferred from the storage vial into an assay vial to make a known concentration for screening/assay purposes. To facilitate the tracking of the rack to be used for the assay vial, post-purification LC/MS and NMR plates, the barcodes from each are scanned into the database. A sample list for the post-purification LC/MS is generated using the HTPP program. Upon the completion of the analysis and processing of the results, the report file is uploaded into the designated network folder and the HTPP program is used to import the results into the database.

The final weight of the compounds for storage and the assay vials are uploaded into the designated network folder and the HTPP program is used to import the weights into the database. At execution of the final step in the HTPP program, an updated Excel, SDfile, and a report file are generated into a.zip folder and notification is sent out to the requestor. A typical output file is shown in Figure 6.

The impact of the integrated mass-directed purification platform, compared to the non-integrated version, is shown in Figure 7. Several of the steps that have been enhanced in the integrated purification platform have led to almost a 50% increase in the throughput. Such steps are the reduction in the time spent in the processing of the data and liquid handling, both in the pre- and post-purification stages. It is estimated that the throughput of a plate of 24 compounds at 50 mg each will take a full time person from the beginning of the process to screening in two days. The numbers in Figure 7 suggest that it is possible for one person to use this integrated purification platform to purify over 12,000 compounds at 50 mg per well in a year.

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Validation and Case Study

To assess the performance and approximate recovery of this platform, we used commercial compounds, described earlier in this paper, and 6 plates, each containing 24 compounds generated from parallel synthesis. One of the issues encountered during the initial testing of the processes is the ability of the analytical chemist to end the process if the crude plate failed QC for any of the reasons listed earlier and re-use of associated fraction collection and vial racks before the end of the processing of a batch. The current program allows for the process to be terminated at any step and the fractions and vials to be reused.

The recovery results from the 18 commercial compounds that had good solubility showed an average recovery of greater than 85% (Table 1) and 70% for the six test plates containing Amgen compounds. The calculation was performed using the formula below:

$$\% \text{ Recovery} = [(\text{final purity} \times \text{final weight})/(\text{crude purity} \times \text{crude weight})]\ 100$$

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Purification Summary

ROW	COL	PURITY	FINAL PURITY	HAYSTACK BARCODE	WEIGHT	ST BARCODE	ST WEIGHT
A	1	100.00	100.00	0101834320	28.46	0032254921	4.1
B	1	89.61	100.00	0101834948	15.79	0032256734	3.28
C	1	100.00	100.00	0101899213	26.54	0032257021	2.98
D	1	100.00	100.00	0101899237	19.14	0032256116	3.13
A	2	100.00	100.00	0101834429	20.09	0032255546	3.87
B	2	100.00	100.00	0101824277	19.1	0032257267	2.38
C	2	100.00	100.00	0101834702	18.21	0032257229	3.51
D	2	100.00	100.00	0101831954	23.33	0032254327	3.14
B	3	81.82	84.22	0101899275	-1.86	0032254426	5.56
C	3	79.25	100.00	0101834856	11.29	0032256024	3.6
D	3	55.45	89.24	0101834832	3.84	0032254983	3.94
A	4	100.00	73.51	0101831701	-0.8	0032257007	4.89
B	4	100.00	100.00	0101831961	17.58	0032255836	2.96
C	4	89.36	100.00	0101832128	5.91	0032255782	3.83
D	4	100.00	90.01	0101831602	12.96	0032254433	4.06
A	5	100.00	100.00	0101831787	20.18	0032256642	3.54
B	5	100.00	100.00	0101831817	14.05	0032254808	3.29
C	5	100.00	100.00	0101831985	16.5	0032256017	4.46

Figure 6. A typical output file from the mass-directed purification platform.

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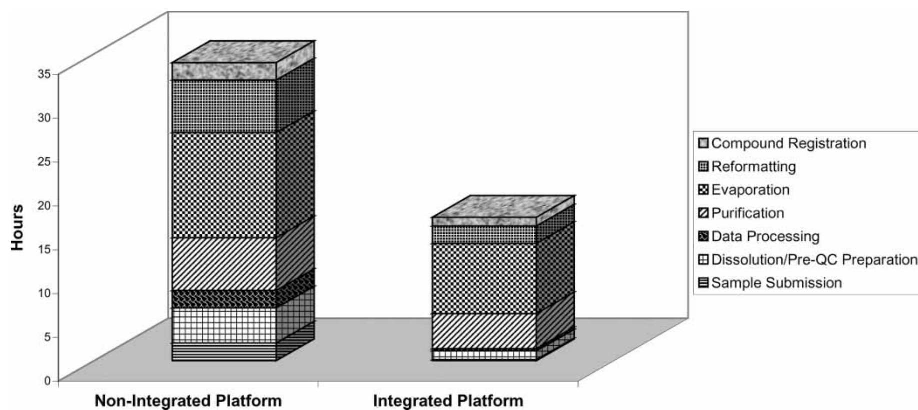

Figure 7. The impact of the integrated mass-directed purification platform when compared to the non-integrated version.

Table 1. The recovery results from the commercial samples (average recovery of compounds that were soluble in methanol was >85%)

Compound	Pre (mg)	Pre purity	Post (mg)	Post purity	Recovery (%)
Dextromethorphan	45	98	43	100	98
Lidocaine	55	99	55	100	102
Amitryptyline	42	99	43	100	120
Trifluoroperazine	48	100	52	100	109
Bendroflumethiazide	50	100	28	100	56
Chlorpromazine	48	100	42	100	89
Terfenadine	43	100	37	100	86
2-Phenybenzoxazole	55	100	23	100	42
Hydroxyzine	54	98	51	100	95
Reserpine	52	97	43	100	85
Trimethoprim	24	100	18	100	75
Thioridazine	41	100	38	100	92
Sulfamethazole	64	99	31	100	49
Benzocaine	50	100	42	100	84
Tolazamide	43	100	3	100	7
Berberine	29	100	23	100	79
Promazine	42	100	50	100	120
Clofazimine	35	98	30	100	87
Average	46	99	36	100	82

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The results of compounds that showed low recoveries could be attributed to the degree of ionization barely above the threshold that was set for these compounds. It was observed that most of the compounds that were purified towards the end of the sequence list showed recoveries greater than 100%. These unusual results were attributed to the concentration of the compounds from evaporation of the solvent used in the dissolution.

Since the implementation of the platform, we have continued to assess the performance of the purification system by routinely determining the recovery of compounds injected onto the system. This assessment is usually performed by dissolving and injecting a known amount of compound, such as reserpine, onto the purification system. The fraction collected is diluted to a known volume in a volumetric flask and analyzed with HPLC-UV by comparing the peak areas of a standard and the diluted fraction. To date, the recoveries on the system have been consistently above 90%.

CONCLUSIONS

A platform has been set up to help speed up the drug discovery process where pure compound ready for assay from a crude plate to storage on a single integrated platform can be produced. The efficient pre- and post-purification

sampling handling, namely, the process for routine plate reformatting and the generation of QC plates for purity assessment by LC/MS and NMR have been presented. The time required for each step, from the receipt of crude compounds for purification to the generation of pure compounds in vials ready for registration, same day generation of screening plates for IC50 assay, and storage, has been reduced by almost 50% when compared to our previous non-integrated platform.

The potential throughput of the platform can be estimated at over 12,000 compounds purified per person at a 50 mg scale per year. At this time, one scientist working on the platform part-time has been able to consistently complete a purification request from crude compound submission to purified compound tested in an assay within 4 days. The steps that ensue when the purified compounds exit our lab are registration and submission into our repository where the assay vials are subsequently diluted to 10 mM and titrated to enable a same day biochemical IC50 screening assay.

This approach and concept is achieved by using a customized electronic compound analysis request program written using Java/J2EE platform and the development of the compound tracking and data handling program written in CSharp (C#) and Microsoft.NET. After the incorporation of these cost effective programs and technologies, the efficiency of the platform is enhanced by the improved data flow throughout the pre-purification, purification, and post-purification stages. For any scientist that uses these programs and these processes, the workstation is friendly, simple, and straightforward.

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