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Mass Spectrometric Techniques for Label-free High-Throughput Screening in Drug Discovery

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High-throughput screening (HTS) is an important tool for finding active compounds to initiate medicinal chemistry programs in pharmaceutical discovery research. Traditional HTS methods rely on fluorescent or radiolabeled reagents and/or coupling assays to permit quantitation of enzymatic target inhibition or activation. Mass spectrometry-based high-throughput screening (MS-HTS) is an alternative that is not susceptible to the limitations imposed by labeling and coupling enzymes. MS-HTS offers a selective and sensitive analytical method for unlabeled substrates and products. Furthermore, method development times are reduced without the need to incorporate labels or coupling assays. MS-HTS also permits screening of targets that are difficult or impossible to screen by other techniques. For example, enzymes that are challenging to purify can lead to the nonspecific detection of structurally similar components of the impure enzyme or matrix of membraneous enzymes. The high selectivity of tandem mass spectrometry (MS/MS) enables these screens to proceed with low levels of background noise to sensitively discover interesting hits even with relatively weak activity. In this article, we describe three techniques that we have adapted for large-scale (~175 000 sample) compound library screening, including four-way parallel multiplexed electrospray liquid chromatography tandem mass spectrometry (MUX-LC/MS/MS), four-way parallel staggered gradient liquid chromatography tandem mass spectrometry (LC/MS/MS), and eight-way staggered flow injection MS/MS following 384-well plate solid-phase extraction (SPE). These methods are capable of analyzing a 384-well plate in 37 min, with typical analysis times of less than 2 h. The quality of the MS-HTS approach is demonstrated herein with screening data from two large-scale screens.

High-throughput screening (HTS) is an important approach for finding active compounds, or "hits", against a therapeutic target of interest. Functional activity-based HTS discovers hits by detecting a change in the activity of a target enzyme in the presence of compounds. Samples containing active compounds contain a relatively high or low concentration of product depending on whether an activator or an inhibitor is present. In either case, large numbers (from 50 000 to over 1 million) of compounds with diverse chemical characteristics are helpful in finding a variety of hits for the following lead compound selection process. As a result, fast analysis times and sample throughput are key to the success of HTS campaigns. Enzymatic functional assays typically use radioisotope or fluorescent labels, allowing rapid measurement of enzymatic reaction products in the sample wells of a high-density microtiter plate. However, labeled substrates may not function exactly the same as native substrates, and coupling reactions used to generate signal can also be affected by compounds. These added interactions can lead to false positives (compounds with active readout but no real activity) and false negatives (active compounds that are undetected). In addition, the synthesis of labeled substrates and the development of coupling reactions may significantly add to method development time and effort.

Mass spectrometry (MS) detects enzymatic products without labeling or coupling reagents, potentially improving data quality and reducing method development efforts. Two published studies on acetylcholinesterase[1] and anthrax lethal factor[2] enzymatic targets have demonstrated the potential of this technique on a smaller scale, and Özbal, et. al from the acetylcholinesterase study have developed an MS-HTS service based on the concept (Biotrove, Woburn, MA). The acetylcholinesterase MS-based screen used a propriety microfluidic system which includes an online chromatographic step prior to introducing samples to an MS. In this study, 4608 compounds were screened in a total of 48 plates (96-well). The anthrax lethal factor study used a SAMDI (self-assembled monolayers for MALDI)-based assay to screen for inhibitors from a library of 10 000 compounds. There have been other label-free techniques which have shown promise for screening as well, including surface plasmon resonance (SPR),[3] desorption/ionization on silicon (DIOS),[4] frontal affinity chroma-

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tography−mass spectrometry (FAC−MS),[5,6] microparallel liquid chromatography,[7] and microchip nanospray devices.[8] Unfortunately, to date, none of these techniques have been fully demonstrated with large-scale HTS campaigns (>10 000 compounds). One contributing factor to the lack of large-scale studies is that instrumentation allowing unattended, fully automated analysis of thousands of samples per day with automated data analysis is not readily available at this time. Also, most MS methods require some type of sample preparation to reduce ion suppression and the resulting poor detection sensitivity.

Liquid chromatography (LC) is the most commonly used approach for separating analytes from sample matrix prior to MS analysis. Tandem MS (MS/MS), specifically using triple-quadrupole detection, has also demonstrated its utility as a highly selective and sensitive method for MS quantitation of enzymatic reactions.[9,10] There have been many techniques for increasing the throughput of LC/MS/MS analyses, especially in the drug metabolism and pharmacokinetics areas of expertise.[11] One example is staggered parallel HPLC, in which four LC runs are staggered in time allowing peaks to be sequentially introduced to the MS/MS to reduce cycle times to 1.4 min per sample with potential for even shorter times.[12,13] Multiplexed (MUX) electrospray is a different approach which has a four- or eight-channel electrospray ionization source interface for analyzing streams from four or eight parallel HPLC systems.[14,15] Analysis times of down to 30 s have been reported using four parallel HPLCs with 1152 samples analyzed in less than 10 h.[16] An even faster approach for introducing samples to the mass spectrometer is high-throughput flow injection in which a 96-sample microtiter plate can be analyzed in approximately 5 min or 3.1 s per sample.[17,18] Still, a sample preparation step is necessary to remove salts and buffer to avoid ion suppression of analytes, and little work has been performed in a 384-well or higher density plate format.[19]

Despite these advances, MS is still considered a medium-throughput technique in which 384-well plates are analyzed in several hours with relatively little automation. Herein, we describe three different techniques that we have developed for HTS of a large compound library (approximately 175K analyses). Both staggered parallel gradient and MUX-LC/MS/MS were developed for running times of approximately 2 h per 384-well plate, allowing unattended overnight analyses. In addition, we describe a newly developed method using eight-way parallel, staggered flow injection MS following 384-well solid-phase extraction (SPE) offline sample preparation to analyze a plate in 37 min. Finally, two inhibitor screening campaign examples are presented. Both targets were previously screened for inhibitors using traditional methods which generated relatively few hits. The primary screen sample decks (~175K wells) were analyzed in either 6 weeks (parallel staggered gradients) or 4 weeks (four-way MUX) using two LC/MS/MS instruments, demonstrating that HTS by LC/MS/MS is practical for large-scale enzymatic functional screens.

MATERIALS AND METHODS

Chemicals. ACS plus grade ammonium hydroxide and sodium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA), as was HPLC grade 2-propanol (isopropyl alcohol, IPA), *n*-pentanol, and acetonitrile. HEPES, DMSO, dimethylisopropylamine (DMIPA), dithiothreitol (DTT), magnesium chloride, sodium acetate, potassium citrate, sodium bicarbonate, and glyceryl triolein (TriO) were obtained from Sigma Aldrich (Atlanta, GA). Glacial acetic acid was obtained from ACROS (Geel, Belgium). 1,1,1-^{13}C-Glyceryl triolein (^{13}C-TriO) internal standard was acquired from Cambridge Isotope Laboratories (Andover, MA). Water was obtained from an in-house Milli-Q Synthesis A10 water polishing system (Millipore, Bedford, MA).

Samples to simulate screening conditions contained the following buffer: 50 mM HEPES pH 7.5, 50 mM $MgCl_2$, 10% isopropyl alcohol (v/v), 4% DMSO (v/v), 10 mM sodium acetate, 2% (v/v) acetic acid, and 1 μM 3-^{13}C-TriO. Samples were prepared by on-plate liquid−liquid extraction in which 50 μL of pentanol was added to 60 μL of sample for each well using a Matrix (Hudson, NH) Wellmate. Plates were sealed using an Abgene (Rochester, NY) thermosealer.

Instrumentation. The following general instrumentation setup was used in all three methods, and detailed conditions are noted as needed with each method description. Abgene (Rochester, NY) polypropylene 384-well plates were used for all experiments and were either normal or deep well format. A Gilson (Middleton, WI) 215/889 eight-way parallel autosampler with either four or eight injectors was used to inject samples and trigger the start of all analyses. Only single injections are necessary for each screening data point due to the highly precise data (<3% CV (coefficient of variation)) obtained while using an appropriate internal standard. A Gilson 10-plate capacity Peltier temperature-cooled sample bed was also used in the autosampler. The autosampler was setup to perform injection port and needle washes during the analysis while resulting in insignificant sample-to-sample carryover (<5%). Each MS-HTS method used Agilent (Palo Alto, CA) 1100 Binary HPLC pumps that were modified in-house with 10 μL micromixers (The Lee Company, Westbrook, CT) to reduce gradient mixing times. Fixed flow splitters were obtained from Analytical Scientific Instruments (El Sobrante, CA) and were used to split flow between the mass spectrometer and waste. A split ratio of 5:1 was used for MUX and parallel staggered LC/MS/MS methods, whereas a 1:1 ratio was used for staggered flow injection MS/MS. Six-port two-way valves (Analytical Sales and Service, Pompton Plains,

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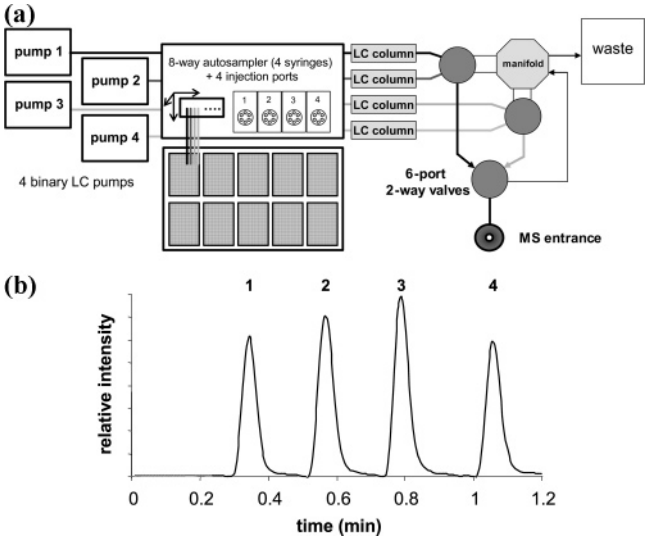

Figure 1. (a) Instrument setup for four-way parallel staggered gradient LC/MS/MS. (b) Total ion current chromatograph obtained using parallel staggered gradient LC/MS/MS. Analyte peaks are serially injected to the MS from four HPLC systems using gradients that are staggered in time, resulting in four sequential analyte peaks in a single chromatogram.

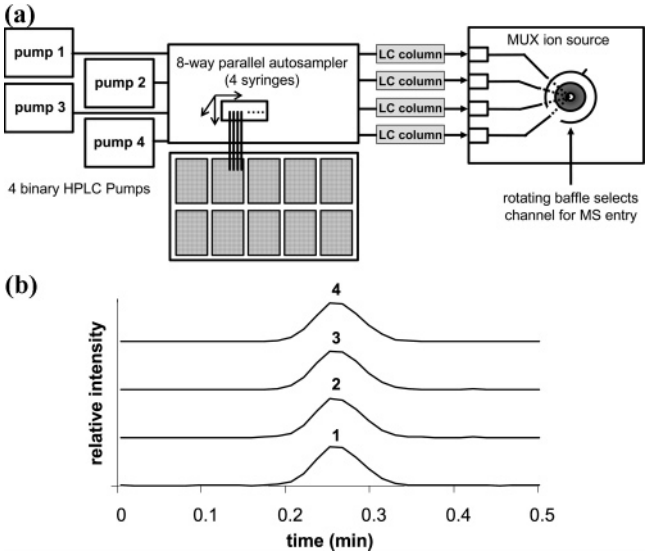

Figure 2. (a) Instrument setup for four-way multiplexed (MUX) electrospray LC/MS/MS. (b) Total ion current chromatograph obtained using MUX-LC/MS/MS. Four parallel separations are monitored by one MS instrument, and four chromatograms are reconstructed by the MS software.

NJ) were used to divert unretained salts and buffers to waste prior to flowing into the mass spectrometer.

Waters (Milford, MA) Quattro Micro triple-quadrupole mass spectrometers were used for multiple reaction monitoring (MRM) analyses in all three HT methods. The following parameters were generally used for all analyses and could be slightly modified based on instrument tuning performance: positive ion electrospray (ESI), ESI capillary voltage = 3 kV, cone voltage = 50 V, source temperature = 125 °C, desolvation temperature = 400 °C, MS1 resolution = 12.5, MS2 resolution = 11.0, collision energy = 26 eV, collision cell Ar pressure = 2.5×10^{-3} bar, mass span = 0.8 Da, interscan delay = 0.02 s, interchannel delay = 0.02 s, MUX interchannel delay 0.03 (where applicable). MRM dwell times ranged from 0.050 to 0.125 s and were chosen for each method to give between 8 and 12 points per chromatographic peak.

All chromatograms were processed using Waters Masslynx 4.0 and Quanlynx software, which calculated the relative response by dividing the area of individual analyte peaks by the area of the 3-^{13}C internal standard peaks. The files of 384 data points were copied as text into Excel where macros were used to (a) deconvolute and label individual peaks from multiple peak files (as in staggered gradients and flow injection methods) and (b) sort the data into a 384-well plate format for export to a LIMS.

Four-Way Parallel Staggered Gradient LC/MS/MS. The LC/MS/MS system consisted of a Gilson 215/889 parallel autosampler using four 500 μL syringes, four independently addressable Rheodyne injection ports, four Agilent binary HPLC pumps, and a Waters Quattro Micro with a single electrospray probe operating in MRM mode. Figure 1a demonstrates the plumbing and layout of this operating mode.

To start, four samples were simultaneously loaded by the autosampler into the four 5 μL injection loops. Full-loop injections of the aqueous samples were injected onto four Thermo (Bellefonte, PA) Betabasic C4 columns (2.1 mm i.d. × 20 mm total length, 5 μm particle size) using the "inject ahead" mode. The isocratic method used mobile phase flowing at 3 mL/min and consisting of 95% methanol and 4% aqueous 0.1% ammonium formate. Columns were replaced after approximately 1500 injections. Injections were triggered by contact closure from one of the HPLC pumps, which is programmed to individually start each of the four injections. The injections were staggered in time so that injection 1 occurred at $t = 0$, injection 2 at $t = 0.25$ min, injection 3 at 0.50 min, and injection 4 at 0.75 min. After peak elution from each individual column, a series of six-port, two-way Rheodyne valves sequentially introduced analyte plugs through a single length of tubing to the MS while diverting the extraneous sample components to waste.

MRM chromatograms of the enzymatic product TriO, 903 > 604 (TriO−NH_4^+, nominal mass) and internal standard, 906 > 606 (3-^{13}C-TriO−NH_4^+) were collected for 1.2 min. The total cycle time for analyzing four samples was 1.5 min (including overhead time from the autosampler and HPLC between runs). Since the analysis is formatted for 384-well plates, a plate was analyzed in 2.4 h.

Four-Way Parallel MUX-LC/MS/MS. Analyses were performed with a Gilson 215/889 parallel autosampler with four 500 μL syringes and four injectors, four Agilent binary HPLC pumps, and a Waters Quattro Micro with a four-way MUX ion source (Figure 2a). To start, four samples were simultaneously loaded by the autosampler, and the MS was triggered as the injection valves were switched from the "load" to "inject" position. For each sample, a 5 μL plug of the organic supernatant, pentanol, from the liquid−liquid extraction was injected onto a Thermo Betabasic C4 guard column (2.1 mm i.d. × 20 mm total length, 5 μm particle size) and eluted isocratically at 1.6 mL/min using mobile phase consisting of 51.5% IPA, 35% acetonitrile, and 13.5% water containing 0.1% ammonium formate. Next, the eluant of each HPLC was split with an ASI 5:1 splitter between waste and the Quattro Micro MS/MS (Figure 2a). Two six-port two-way Rheodyne valves were used to divert the four mobile phase streams to waste for 0.2 min,

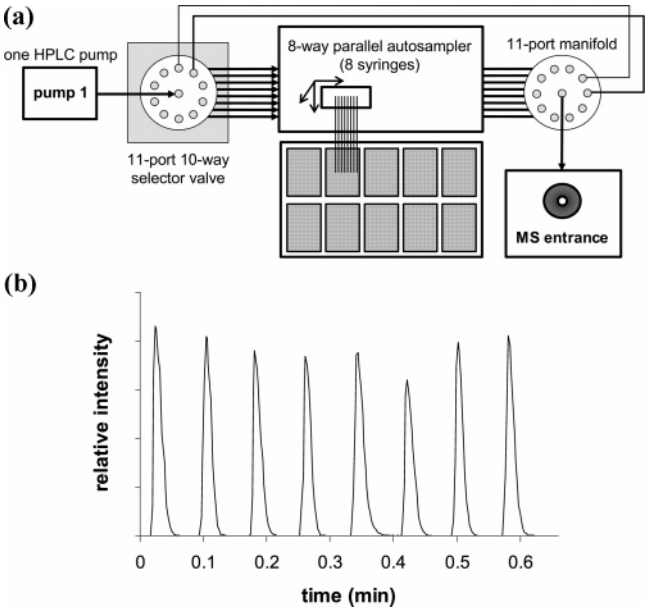

Figure 3. (a) Instrument setup for eight-way serial flow injection MS. (b) Total ion current chromatograph obtained using serial flow injection for samples that have been cleaned up by SPE prior to analysis.

at which time the primary Agilent HPLC pump triggered the valves to direct flow to the mass spectrometer.

MRM chromatograms of 903 > 604 (TriO−NH_4^+, nominal mass) and 906 > 606 (3-^{13}C-TriO−NH_4^+) were collected for a total of 0.55 min (including the 0.2 min solvent divert time). The total cycle time for analyzing four samples was 1.2 min (including overhead time from the autosampler and HPLC between runs). Since the analysis is formatted for 384-well plates, a plate was analyzed in 1.8 h.

Eight-Way Staggered Flow Injection MS with 384-Well SPE. An Orochem Technologies (Lombard, IL) SuPErScreen C18 384-well SPE plate from ChromTech (Apple Valley, MN) was pretreated by sequentially wetting and eluting with 50 μL each of IPA, water, and reaction buffer. The reaction buffer, used to simulate an acid-quenched reaction, consisted of 90% aqueous 50 mM HEPES and 50 mM $MgCl_2$ in 2% glacial acetic acid with 10% IPA. All solvents applied to the SPE plate were eluted into a 384-well collection plate using a Hermle (Labnet International, Woodbridge, NJ) Z383 plate centrifuge for 2 min at 1750 rpm. Following pretreatment, 50 μL samples of 0.5, 0.45, 0.25, and 0.05 μg/mL TriO with 0.5 μg/mL ^{13}C-TriO in the reaction solution were loaded on the plate. After elution, four separate 50 μL water washes were performed to remove the buffer and salts. Lastly, two 50 μL elutions of IPA eluted the analytes and were collected into a deep well plate.

Analyses were performed with a Gilson 215/889 parallel autosampler with eight 500 μL syringes and eight injectors, one Agilent binary HPLC pump, and a Waters Quattro Micro with a single electrospray probe operating in MRM mode (Figure 3a).

The mobile phase consisted of 67.5:25:7.5 (v/v/v) acetonitrile/IPA/aqueous ammonium formate (1 g/L) at 1.5 mL/min flow rate. To start the eight-sample staggered flow injection sequence, the Gilson 215/889 autosampler injected eight samples from a 384-well plate into the eight injection valves in the load position, preparing for a 1.5 μL full-loop injection. A single Agilent 1100 binary pump was used to isocratically pump mobile phase into the first port of a Rheodyne 10-position, 11-port selector valve that followed through the first injection valve on the Gilson. To start the run, all of the autosampler injection valves were triggered into the "inject" position, allowing the first sample to pumped through the sample loop past the 11-port manifold into the single tube. Next, the 11-port valve was triggered to the next position, pumping the next sample plug through the manifold. Mobile phase was sequentially pumped through all eight injection ports on the Gilson 215/889 autosampler moving all of the sample plugs through the manifold into the single tube. Finally, the sample plugs passed sequentially through an ASI 1:1 calibrated splitter, where the eluent was split between waste and the Quattro Micro MS/MS (Figure 3a). Elution of each plug was delayed by 0.08 min to achieve a run time of 0.66 min with 0.77 min cycle time, including instrumentation resets.

The MS collected data in ESI positive mode with two multiple reaction mode (MRM) transitions at 903 > 604 (TriO−NH_4^+, nominal mass) and 906 > 606 (3-^{13}C-TriO−NH_4^+). Total analysis time for a 384-well plate was 37 min.

RESULTS AND DISCUSSION

Methodology. Until recently, throughput has been the major limiting factor of using MS for label-free detection in high-throughput screens. There are several approaches to consider for increasing throughput with existing technology to make MS amenable for HTS. One obvious route is to utilize several instruments for analysis at the same time. Unfortunately, it is expensive and tedious to set up laboratories and maintain many instruments for MS quantitation. In each of the following three approaches, a parallel autosampler reduces the typically rate-limiting cycle time of the autosampler and allows more efficient use of a single mass spectrometer for quantitation of single chromatographic peaks.

Four-Way Parallel Staggered Gradient LC/MS/MS. In this version of a parallel staggered gradient LC/MS/MS approach, four HPLC pumps are necessary, but only one MS and autosampler are required. The processes of injecting, separating, and detecting have been optimized to remove seconds or fractions of seconds from each step, resulting in relatively short analysis times. In addition, the "inject ahead" mode is utilized to load samples into the injector for the next analysis, saving additional injector overhead time during the next run. The HPLC gradient is optimized so that analyte peaks are eluted as quickly as possible after the buffer, salts, and substrates are diverted to waste. Then each of the individual pump gradients is staggered to allow enough time for the analyte and internal standard to elute before switching to the next LC stream. As shown in Figure 1b, four samples are analyzed in 1.2 min. As the analysis of four chromatograms completes, the data is saved by the computer and the system triggers the next run, resulting in a 1.5 min cycle time for four sample analyses and 2.4 h for a 384-well plate.

Four-Way Parallel MUX-LC/MS/MS. Similar to the previous approach, four-way MUX-LC/MS/MS also uses four HPLC pumps with one parallel autosampler and one MS. In this case, the MUX interface alternates MS detection between each of the streams of the four HPLCs, allowing each of the separations to occur

Table 1. Relative Comparison of Three MS-HTS Methods

	sensitivity	speed	reagent cost
staggered parallel gradients LC/MS/MS	high	slow	moderate
multiplexed LC/MS/MS	low	moderate	moderate
staggered flow injection MS/MS w/384 well SPE	moderate	high	high

concurrently. The detection time is shared between four channels with delays occurring between each channel. As a result, there is a loss in sensitivity when using MUX analysis. For TriO, this loss was 8-fold when comparing a 0.5 s single-channel dwell time with a 0.1 s dwell time on the four-channel MUX. The chromatograms in Figure 2b demonstrate the fast separation times (less than 30 s), as well as the interchannel reproducibility of the four HPLC runs. In this mode, however, it is not possible to use the "inject ahead" function due to the process of deconvoluting the MUX files into four sequential samples in the sample list. As a result, the total cycle time for four injections is approximately 1.2 min, including 0.65 min of overhead time. This results in a 384-well plate analysis time of 1.8 h.

Eight-Way Staggered Flow Injection with 384-Well SPE. Unlike the previous approaches, there is no HPLC separation step in the flow injection approach, and ion suppression is likely to occur from coeluting buffers, salts, and compounds present in HTS samples. As a result, some sample preparation is necessary prior to MS analysis. SPE is commonly used to separate analytes from a matrix but is typically performed in singles or in 96-well plates. Though not commonly available, 384-well SPE plates have the potential to increase the throughput and efficiency of SPE in the screening process. Figure 3b contains a series of flow injections of TriO that have been extracted by 384-well SPE from screening samples. The analysis time is determined by individual peak widths and the delays that are included to ensure reproducible peak integration of adjacent peaks. In this case, an analysis time of 0.66 min (40 s) was necessary for eight sample analyses. Similar to the staggered parallel gradient method, injector overhead time is reduced by using the "inject ahead" function, and the overhead time is shared by eight samples instead of four. This results in a total cycle time of 0.77 min and a relatively fast 384-well plate analysis time of only 37 min.

Choosing a Method. When developing a method for an MS screen using one of these three methods, several factors are considered including sensitivity, throughput, and cost. These factors are summarized in Table 1 comparing only the three methods. The MUX method is typically faster than staggered serial gradients since delay times between LC streams are not necessary. However, MUX is undoubtedly less sensitive for a given HPLC condition since MS sampling times are shared between four channels. If MS throughput is the ultimate goal, then staggered flow injection is the fastest of these three methods. With a well-developed SPE method, sensitivity of this method can approach that of LC-based methods. Unfortunately, at this time, reagent cost per well is significantly higher using 384-well SPE plates than using columns since a typical column can last over 1000 injections and cost significantly less than an SPE plate. Of course, there are other cost factors such as MS instrument time and liquid handling that should also be considered.

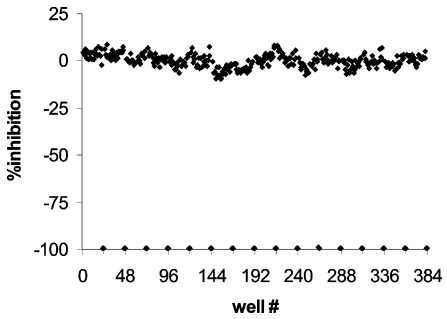

Figure 4. Reaction control plate containing TriO analyzed by MUX-LC/MS/MS. Data was obtained from a 384-well plate of uninhibited enzymatic reaction controls (columns 1−23) and no-enzyme controls (column 24). Data is presented sequentially from wells 1−384 (corresponding to A1−A24, B1−24, through P24).

Large-Scale Screening with LC/MS/MS. The 384-well plates are arranged with compounds in columns 1−22, a high (uninhibited) control in column 23, and a low control (without enzyme) in column 24. The relative response of individual wells is converted to percent inhibition based on the high and low controls. Because of the many liquid-handling steps involved, a quality control (QC) plate is prepared without compounds to check the consistency of data across all wells of the plate. Figure 4 contains representative data from a QC plate from the HTS campaign which used the MUX technique for TriO analysis. In this case, a CV of 3.5% occurred between uninhibited wells, demonstrating the reproducibility of the pipetting and analysis as well as the enzymatic reaction across the plate. A common statistical tool, Z', is used to demonstrate the dynamic range, the variation of data, and the separation that is expected between uninhibited and inhibited enzymatic reactions on the plate.[20] An ideal assay has a $Z' = 1$, and an excellent assay has a value between 0.5 and 1.[20] The assay in Figure 4 has a Z' of 0.89, demonstrating the quality of assay that is possible using MS.

To further demonstrate the quality of the assay, a blind test plate was prepared in which 15 wells were spiked with one of three levels of inhibitor, corresponding to 10%, 50%, and 90% inhibition. Figure 5 contains a graphical representation of the 384-well plate, where the shading represents the percent inhibition of a well. The 90% and 50% inhibitors were easily distinguished, whereas the 10% inhibitors were identified with an additional four false positive results.

Several HTS campaigns of 100−200K wells have been conducted using the methods described herein, including some that were not possible to screen with traditional screening techniques. Figure 6, parts a and b, contains the Z' values of 384-well plates representing a total of over 332 500 samples for (a) a primary screen using the MUX method for TriO and (b) a primary screen using the staggered parallel gradient method on an undisclosed analyte. In both cases, the average plate Z' for the entire screen was greater than 0.7, with a few outliers having Z' less than 0.5. Minor patterns were present indicating that issues may have occurred with the starting solutions, liquid handlers, and/or LC/

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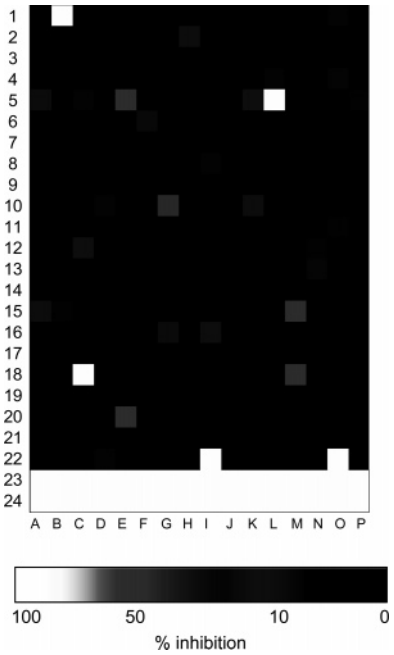

Figure 5. Blind test plate containing uninhibited reaction controls (columns 1−22), no-enzyme controls (rows 23 and 24) with five wells each of 90%, 50%, and 10% inhibition. Wells of 90% and 50% inhibition are found with 100% accuracy. Although all five 10% inhibitors are found, there are also four false positive hits.

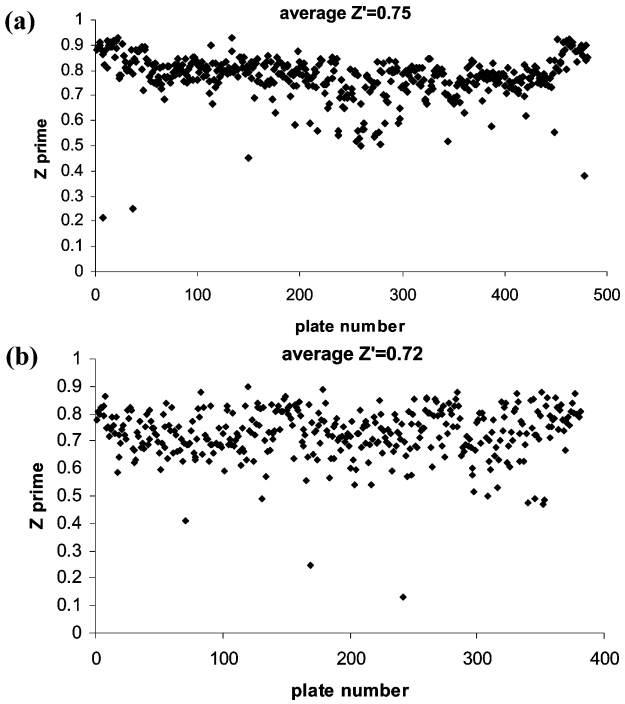

Figure 6. Performance of two primary screening campaigns. Each data point represents the *Z′* of a 384-well plate. (a) Plate *Z′*s of a primary screen which utilized MUX-LC/MS/MS. (b) Plate *Z′*s of a primary screen which utilized serial parallel gradients LC/MS/MS.

MS/MS instruments. Furthermore, outliers are not unexpected with the many steps that occur, and it is possible to repeat plates when *Z′* values are insufficient for inhibitor identification. The analysis of each of these two large-scale screens were conducted in less than 6 weeks using two automated instruments capable of running unattended for 24 h.

In comparison to ultra-high throughput screening methods (>100 000 wells/day),[21] these MS methods are relatively slow (<10 000 wells/day), making single compound per well screening of >1 million compounds impractical at this time. To enable entire library screening, we utilize orthogonally prepared mixtures of 10 compounds per well in which each compound is located in two wells of unique compound composition. A compound is selected as active only if it demonstrates activity in both wells. This strategy results in a 5-fold improvement of throughput with similar risk of false readouts.[22] Additional time savings may be realized since the following common procedures are unnecessary: (a) custom labeling of radiometric and fluorometric reagents, (b) selecting and developing methods for coupling assays, (c) counterscreening of coupling enzymes, and (d) counterscreening to identify false positives from inherent compound absorbance or fluorescence. Although the MS analysis is relatively slow, overall timelines of MS-based screens from concept to hit list are similar to those of traditional HTS and uHTS-based screens.

Although the cost of using any detection mode varies among targets, reagent and instrumentation costs of MS screening are currently similar to other 384-well plate methods since labels and coupling assays are unnecessary. Still, the relatively long analysis times and 384-well format result in more researcher time and larger reagent volumes when compared with 1536-well-based uHTS methods. As a result, MS-HTS is typically more expensive and selected for opportunities when other HTS techniques are difficult, impossible, or significantly more expensive due to specialized reagents. For example, MS screens can be conducted using impure enzymes or homogenates with mixed substrates. Although LC/MS is typically less sensitive than fluorescence and radiography, its high selectivity may enable an assay with matrix or compound interference in typical assays. In one of the cases demonstrated in this article, MS provided a more sensitive assay than the previously utilized absorbance-based assay and many novel hits were identified, providing new and diverse chemotypes for the drug discovery program.

Overall, MS-HTS is a powerful technique that can be used for most screens in which there is an enzymatic conversion of substrate to product. Still, there are target classes in which traditional screening methods are well established and less expensive. Therefore, until technology enables faster, cheaper MS analysis to compete with uHTS techniques, MS-HTS will likely continue to be used in opportunities where it is the best of all screening options.

SUMMARY

Mass spectrometry is a label-free analysis technique that can be used for measuring enzymatic reactions in high-throughput screens. Three unique methods using the same instrument components have been described herein and are chosen for screening based on throughput, sensitivity, and cost. High-quality screening data has been shown from two MS-based primary

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(22) Ferrand, S.; Schmid, A.; Engeloch, C.; Glickman, J. F. *Assay Drug Dev. Technol.* **2005**, *3*, 413−424.

screens, demonstrating the practicality of performing label-free high-throughput screens with MS. MS-HTS is an important tool in drug discovery for screening targets that are difficult or impossible with traditional HTS methodology. With further development of MS technology in the near future to improve throughput, MS screening may become a mainstream tool in high-throughput drug discovery.

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