Special Report

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Bioanalysis

Perspective on high-throughput bioanalysis to support *in vitro* assays in early drug discovery

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As the desire for a shortened design/make/test/learn cycle increases in early drug discovery, the pressure to rapidly deliver drug metabolism pharmacokinetic data continues to rise. From a bioanalytical standpoint, *in vitro* assays are challenging because they are amenable to automation and thus capable of generating a high number of samples for analysis. To keep up with analysis demands, automated method development workflows, rapid sample analysis approaches and efficient data analysis software must be utilized. This work provides an outline of how we implemented those three aspects to provide bioanalytical support for *in vitro* drug metabolism pharmacokinetic assays, which include developing hundreds of mass spectrometry methods and analyzing thousands of samples per week, while delivering a median bioanalytical turnaround time of 1–2 business days.

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The rising costs of drug discovery and development have been well documented, and correspondingly the pressure on discovery organizations to efficiently generate discriminatory data has also increased [1–3]. The mantras of 'fail early' and 'fail fast' have become ubiquitous within the drug discovery culture, including within drug metabolism pharmacokinetic (DMPK) groups across the industry. These influences, along with the ease of use of many automated liquid handling platforms, have led to greater implementation of moderate- to high-throughput *in vitro* DMPK assays, even to the point of testing each compound made within a discovery organization in certain key assays (defined as 'tier 1 assays' moving forward) [1,2,4–6]. These assays generate data that can be used for multiparametric optimization or potentially the *in situ* prediction of key parameters based on large datasets [1,6–13].

When considering these moderate- to high-throughput *in vitro* assays, there is an array of combinations that can be implemented with respect to where/how compounds are made (chemistry), assayed (*in vitro*), analyzed (bioanalysis) and processed/reviewed. The options available go so far as to include the automated liquid handler used to perform the assay or the analytical approach taken during sample analysis. Multiple options are available for automation from vendors such as Tecan, Hamilton and Perkin Elmer depending on the user requirements [5,6,14–18]. Similarly, there are multiple mass spectrometer vendors that provide instrumentation across potential analytical techniques or configurations (e.g., online solid phase extraction, ultra performance liquid chromatography [UPLC] and acoustic ejection), each having its own strengths and limitations that have been discussed in the literature [2,4–6,19–30].

Based on these different options, the 'right' selection for a given situation will be organization specific, though three common criteria typically dictate the final strategy: cost, quality and speed. Cost pertains either to the lump-sum price of having a contract research organization (CRO) perform the work or to the aspects that go into internally performed assays (e.g., salary, consumables, instrument time). Quality refers not only to the specific data generated but also to the level of oversight and long-term consistency achievable. Lastly, speed is defined by the turnaround time (TAT) from assay request to data availability, including shipping duration.

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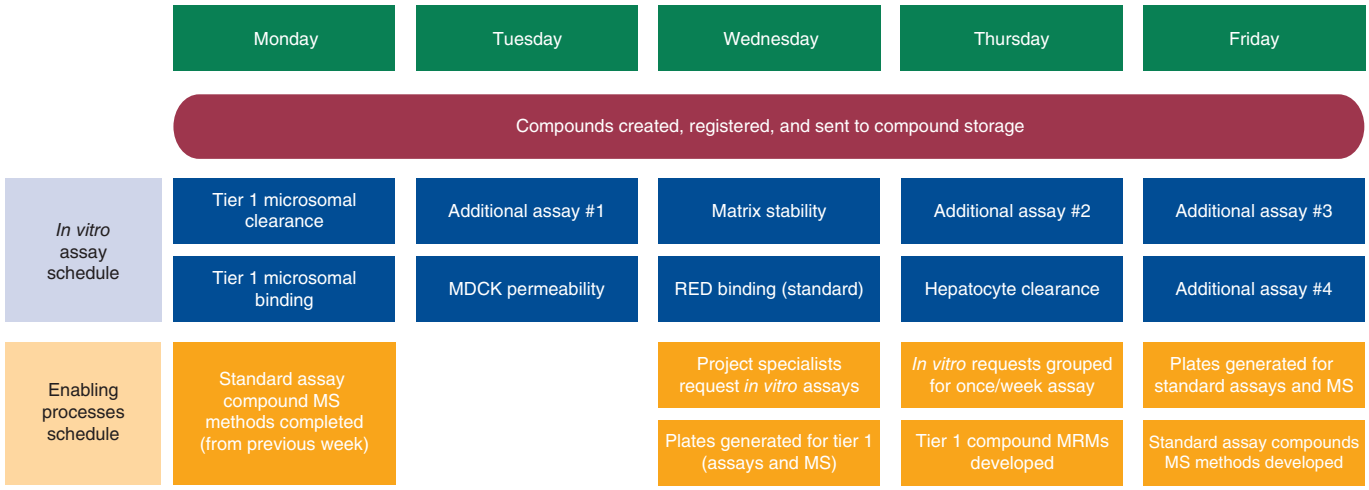

Figure 1. Depiction of the weekly drug metabolism pharmacokinetic workflow. The figure includes a typical *in vitro* assay schedule (blue markers) and timeline for the assay-enabling processes (orange markers), along with medicinal chemistry processes shown as continually ongoing (dark red marker).
MDCK: Madin-Darby canine kidney; MRM: Multiple reaction monitoring.

This last point regarding shipping duration is particularly impactful given how it frames decision-making with respect to all three criteria for any global company, especially those that have widely distributed research sites or employ CROs. These companies are faced with an unavoidable decision regarding the balance of speed, quality and the cost required to optimize these key criteria. Prioritizing speed by setting up/running assays at all research sites (including CROs) requires that significant effort be made in proving the assays are equivalent, or else there is a risk that the data quality will be impacted. Focusing on quality requires the increased efforts mentioned above, which raises cost due to additional controls (scientific and logistic) or reduces speed due to the need to ship compounds to a single hub for a given assay. Regardless of the systems put in place, shipping a compound between sites will always be slower than moving it between laboratories at the same facility.

Different iterations of assay and bioanalytical workflows have been previously shown in the literature [4,6,31]. A summary of the weekly workflow we have implemented is shown in Figure 1, where an example *in vitro* schedule is displayed using blue markers and the enabling processes are shown with orange markers. A key decision point within the overall workflow is the choice to plate compounds and develop MS methods once per week for each assay type (tier 1 and standard). Additionally, assays are only performed once per week, including the tier 1 assays, standard DMPK *in vitro* assays (e.g., matrix stability, Madin-Darby canine kidney [MDCK] cell permeability, protein binding) and additional *in vitro* assays run on an as-requested basis (e.g., blood-to-plasma ratio, hepatocyte uptake). It was determined that the potential improvement in TAT accomplished by performing these assays multiple times in a week (speed) was not worth the efficiency loss (cost) caused by the duplication of efforts.

To provide the bioanalysis required by this type of large-scale *in vitro* DMPK support, we assessed the hardware and software available across the industry, focusing on aspects that would be easily employed with our existing fleet of instruments while providing suitable speed and quality at an acceptable cost. Although the bioanalytical practices described herein have generally been reported in the literature, we are sharing our perspective on why and how we implemented them. By supplying the overall rationale of why these bioanalytical approaches were taken, while also giving specific examples based on our experiences and practices (e.g., how we tailor the analytical approach to the assay requirements and the number of samples to be analyzed), we look to assist others as they attempt to maximize on the three key performance markers of cost, quality and speed in the same way that we have.

Materials & methods

Compounds submitted for assays are grouped once weekly based on assay tier (tier 1 or standard) and plated in 96-well-plate format by GSK Sample Management. Each well contains a single compound dissolved at 10 mM in DMSO and aliquoted at a volume of 40 µl. Upon receipt, samples are diluted for MS/MS method development using a Tecan Evo200 (Männedorf, Switzerland) (v. 2.08) to 50 µM, and then to a final concentration of 400 nM in 80:20 acetonitrile:water containing 0.1% formic acid.

Table 1. Mass spectrometry source parameters for Sciex instruments used to analyze tier 1 and standard drug metabolism pharmacokinetic *in vitro* assays.

Mass spectrometer:	Sciex 5000 QQQ	Sciex 5500 QTRAP	Sciex 6500 QQQ
Scan type	MRM	MRM	MRM
Resolution Q1	Unit	Unit	Unit
Resolution Q3	Unit	Unit	Unit
CUR	40	35	40
IS (positive)	4000	4000	4000
IS (negative)	-4000	-4000	-4000
Source temperature (°C)	600	600	450
GS1	50	60	65
GS2	50	45	65
CAD	7	9	7

CAD: Collision Gas; CUR: Curtain Gas; GS: Gas Setting; IS: Ion Spray; MRM: Multiple reaction monitoring.

LeadScape® software from Sound Analytics (CT, USA) is currently used to determine the multiple reaction monitoring (MRM) and compound-specific MS parameters for each compound to be analyzed (both tier 1 and standard assays). The 400-nM samples are infused via the conditions shown in Supplementary Table 1 using an LS-1 autosampler coupled to a Sciex (MA, USA) 5500 QTRAP®, and the decision tree in Figure 2 shows which steps are taken for MRM development for each compound. Additionally, Figure 3 shows the workflow for how the ChromaTune aspect of LeadScape® is used to test generic chromatographic conditions for each compound to be used in standard *in vitro* assays. The generic MS source conditions are shown in Table 1, and chromatographic settings for the ChromaTune process are shown in Supplementary Table 2. For compounds that display overly high signal during the initial Q1 scan of the QuickTune process, the signal is reduced using the software's built-in saturation control feature, allowing for improved method accuracy with respect to precursor mass-to-charge ratio and declustering potential. Non-compound-specific MS source conditions are applied for all *in vitro* analyses but are specific to the instrument type being used. Electrospray ionization is used as the ionization technique, and equivalent but opposite instrument settings are used for positive and negative modes.

Reverse-phase liquid chromatography is performed across instruments (various UPLC systems and autosamplers) using two different condition groupings, as detailed in Supplementary Table 3. Water with 0.1% formic acid (A1) and acetonitrile with 0.1% formic acid (B1) are used as the standard mobile phases; however, water with 0.1% ammonium hydroxide (A2) and acetonitrile (B2) are also kept at all instruments and used when unsuitable chromatography is observed under acidic conditions. Additionally, for compounds with little or no retention, the parameters in Supplementary Table 3 are modified such that the mobile phase is initially 100% aqueous. Sample injection volumes are typically 1–4 µl, depending on the assay and instrument combination, with actual sample consumption estimated to be 1–9 µl depending on the autosampler model used.

Two LS-1 autosamplers from Sound Analytics coupled to Sciex 5500 QTRAP mass spectrometers are used in trap-and-elute mode for tier 1 assay analysis. Each system utilizes four ExionLC™ AD UPLC pumps (Sciex), two Exion 5 channel degassers (Sciex) and two Shimadzu (Kyoto, Japan) CBM-20Alite system controllers. The autosamplers utilize a Polystyrene-divinylbenzene (PS-DVB) 1.5 × 5 mm trap column for all studies, with water containing 0.1% formic acid as the aqueous (loading) phase and 90% acetonitrile containing 0.1% formic acid as the organic (eluent) phase. The system liquid is water with 0.1% formic acid, and the wash solvent is 50:50 water:acetonitrile. The system uses a 2-µl injection loop and consumes an estimated 15 µl of sample per injection due to the internal volumes of the system. During initial experiments, the instruments were set to make an injection every 20 s using the timings shown in Supplementary Table 4. The current settings are shown in Supplementary Table 5; they allow for an injection to be made every 12 s.

MultiQuant™ (v. 3.0, Sciex) is utilized for all data analyses. Using the embedded query functionality and custom Excel-based logic, datasets are interrogated for the key analytical parameters of analyte signal-to-background versus the matrix blank, internal standard signal variation, analyte and internal standard peak height, and analyte and internal standard peak integration quality. The Excel logic is set to highlight samples that fail these criteria, facilitating rapid troubleshooting for standard assay datasets and allowing for a review-by-exception workflow for the tier 1 assays.

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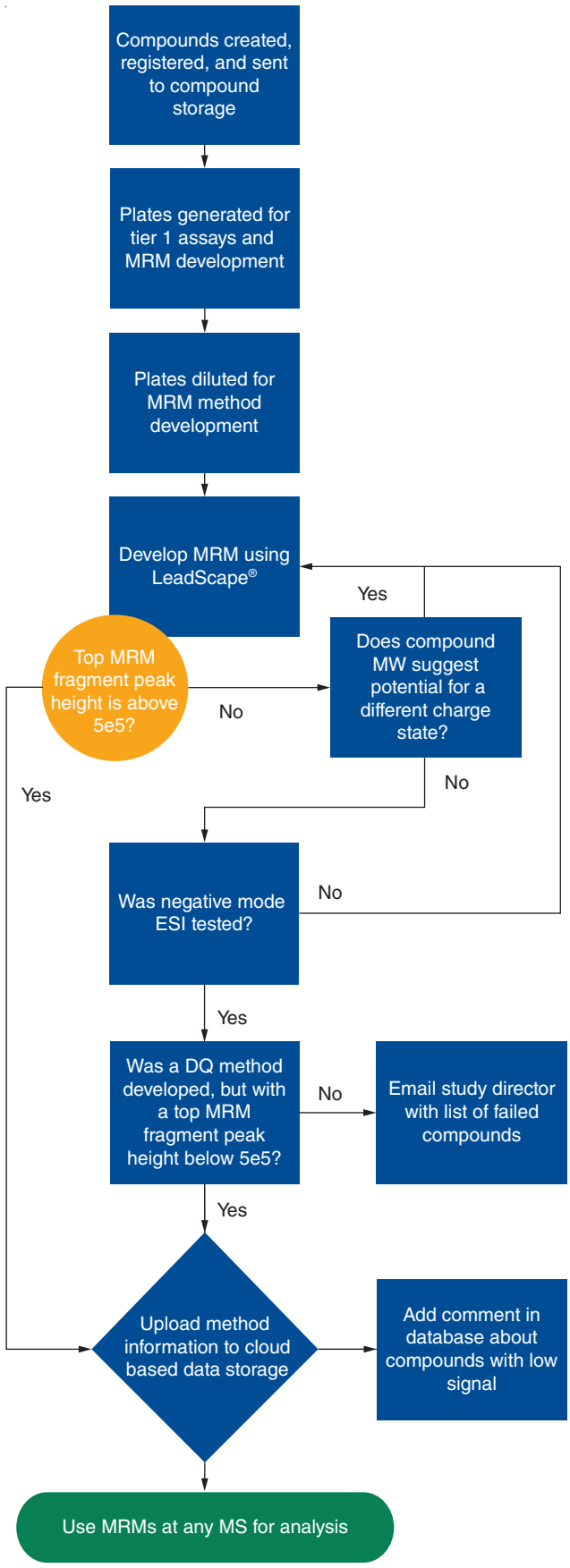

Figure 2. Workflow used to determine the multiple reaction monitoring method for every compound in the drug metabolism pharmacokinetic tier 1 and standard *in vitro* assays. LeadScape® software and an LS-1 autosampler are used with a Sciex 5500 QTRAP® instrument, and final methods are uploaded to a globally assessable database.
MRM: Multiple reaction monitoring; MW: Molecular weight; ESI: Electrospray ionization; DQ: DiscoveryQuant™; MS: Mass spectrometer.

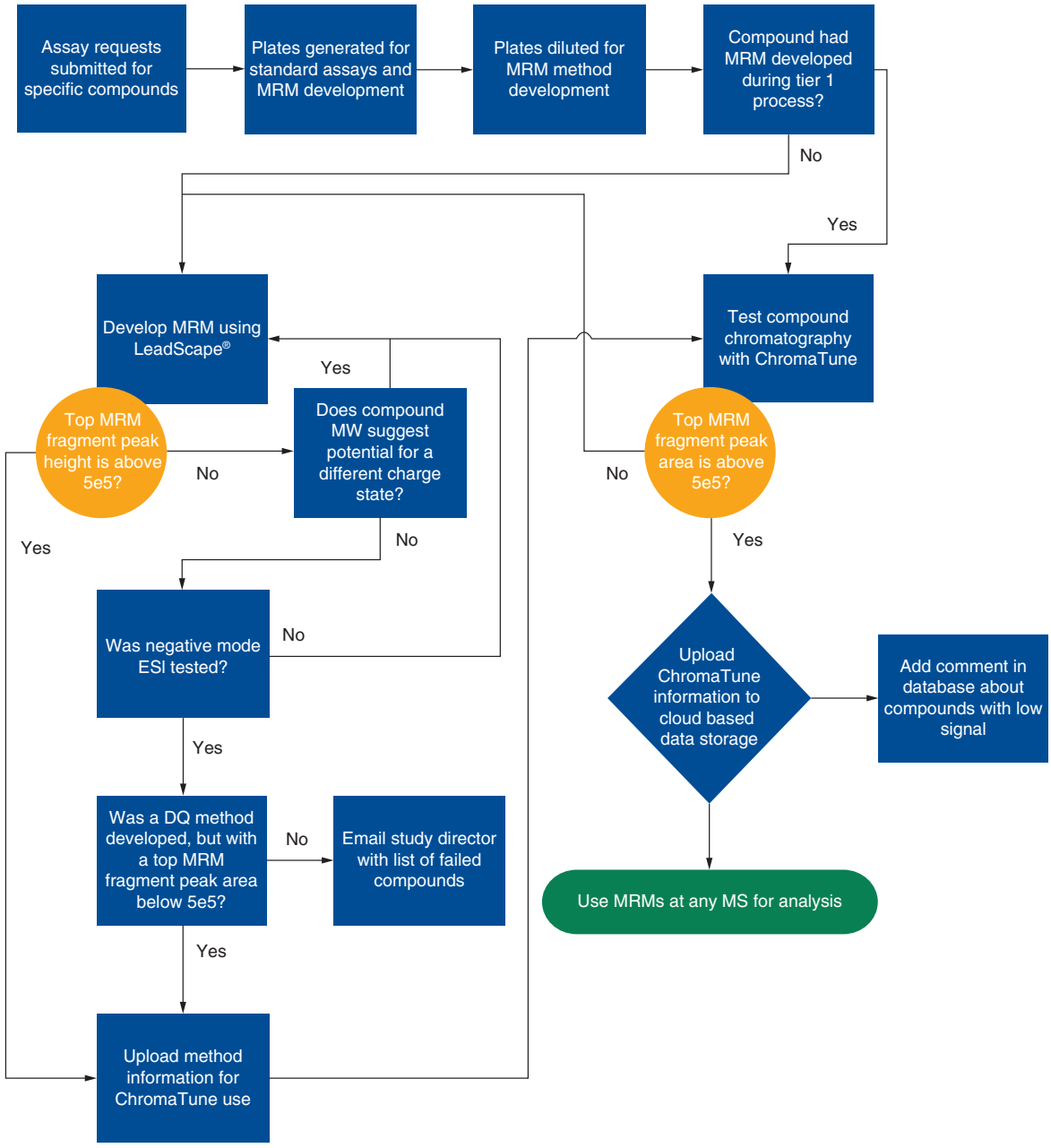

Figure 3. Depiction of the ChromaTune generic high-pressure liquid chromatography condition testing workflow, including developing a compound multiple reaction monitoring for compounds without a previously determined method. LeadScape® software and an LS-1 autosampler are used with a Sciex 5500 QTRAP® instrument, and final results are uploaded to a globally assessable database.
MRM: Multiple reaction monitoring; MW: Molecular weight; ESI: Electrospray ionization; DQ: DiscoveryQuant™; MS: Mass spectrometer.

Two Tecan Fluent® 1080 automated liquid handlers are used to perform the weekly tier 1 assays. Each assay is supplied with its specific set of 96-well plates by GSK Sample Management, which are diluted and then aliquoted to the appropriate wells using the Fluent MultiChannel Arm™ 96-head adapter. The microsomal binding is performed using rapid equilibrium device (RED) plates with an incubation period of 4 h. Receiver (buffer) and donor (microsomal incubation) aliquots from the RED plates are matrix-matched using blank buffer/matrix. Microsomal clearance wells are sampled at five time points, ending at 45 min. Final samples from the tier 1 assays are aliquoted into 384-well plates and protein-precipitated using acetonitrile containing the internal standard

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fexofenadine (200 ng/ml) at a sample:acetonitrile ratio of either 40:120 µl (microsomal binding) or 30:125 µl (microsomal clearance).

A Tecan Fluent 1080 is also used for the standard 4-h RED protein binding assay, utilizing a modified script capable of flexibly mixing and matching compounds and matrices (plasma, blood, microsomes, media, buffer) across species. Receiver (buffer) and donor (binding matrix) aliquots from the RED plates are matrix-matched using blank buffer/matrix. The final samples from this assay are transferred and protein-precipitated as described above for the tier 1 binding assay. All other *in vitro* assays are performed manually, and final samples are provided at a sample:acetonitrile ratio of 1:4, with total sample volume being specific to the assay. The acetonitrile used for protein precipitation contains the internal standard fexofenadine at 200 ng/ml for all assays; however, 200 ng/ml labetalol is also added to the MDCK and RED crash solutions as an alternative internal standard because matrix effects have previously been observed to impact fexofenadine signal in these assays. Separating the *in vitro* assay and bioanalysis with the protein-precipitation step allows optimal conditions to be achieved for both aspects, such as plate temperature and mixing for the *in vitro* assays and minimal protein concentration in the injected bioanalytical sample.

Initially, *in vitro* assay result processing and visualization were performed using custom Microsoft Excel®-based workbooks. Bioanalytical data were imported as .txt files, including the MultiQuant Query calculation/output columns. Rate constants were calculated for clearance/stability assays using peak area ratio data fitted by the XLfit functionality embedded within the software. Other assay calculations (e.g., free fraction, permeability) were also performed within the Excel workbook. Subsequently, Signals™ VitroVivo (Perkin Elmer, MA, USA) was implemented as a data analytics platform to replace Excel and enable integrated secondary assay calculations and curve fitting. Exports from the platforms are uploaded directly to Perkin Elmer's Signals electronic notebook.

Discussion

In this section we will highlight the specific approaches we have taken to LeadScape®/DiscoveryQuant™ use and the analysis of tier 1 and standard *in vitro* assays, which have allowed us to maintain a 1- to 2-day median bioanalytical TAT for *in vitro* assays over the past two calendar years. The decision tree depicted in Figure 4 highlights the bioanalytical instrumentation and methodologies used to support higher-throughput tier 1 assays (hundreds of compounds) and standard weekly DMPK *in vitro* assays (tens of compounds). Details about how these decision points were made are contained below, along with relevant information about the software packages that also helped enable the current workflows. Although alternative workflows are certainly available, we feel this process consistently provides high-quality data within a rapid TAT (speed), without the need for overly complex or niche automation/instrumentation, or automation/instrumentation that is still under development (cost).

LeadScape® for automated method development

One pivotal aspect of the bioanalytical workflow that impacts the speed, quality and cost of every assay is the MS method development. Unlike many screening assays where one or a few ligands are monitored in every sample, the relative amount of each unique compound is measured in our assays. To handle the MRM method development volume on a week-by-week basis, an automated solution is required (speed, cost) [5,32–34]. Although options exist from different vendors [4,6,21,29,30,34,35], based on our experience with Sciex instruments we chose to utilize their DiscoveryQuant software platform, which determines the compound MRM settings (e.g., parent mass-to-charge ratio) and optimizes the MS source/collision cell conditions (e.g., declustering potential, collision energy) for individual compounds through a flow injection analysis experiment. Upon transitioning this process to an instrument with an LS-1 autosampler, the shift was also made to using the LS-1-specific software LeadScape®. With respect to MRM method development, the two software packages (both originally developed by Sound Analytics) function very similarly from a user perspective; however, the LS-1 allows for a faster method development due to its open-deck layout and rapid sampling speed. For both software packages, the data can be reviewed and uploaded to a centralized SQL database following the method optimization. This enables compound-specific parameters to be stored in a table format that can be pulled down to each of the instruments within the lab (or connected lab sites).

In our laboratory, a 20-µl injection is made to the mass spectrometer using a columnless line, resulting in a broad peak (15–18 s wide) that allows for MS method optimization of the different parameters at an assumed 'steady state' of signal. This process is defined as 'MRM Only' within the software and can be performed at two levels of rigor, depending on the level of optimization needed. We currently perform both the available quick and fine-tune levels (quality), as the developed methods are used across tier 1, standard *in vitro* and *in vivo* analyses, the

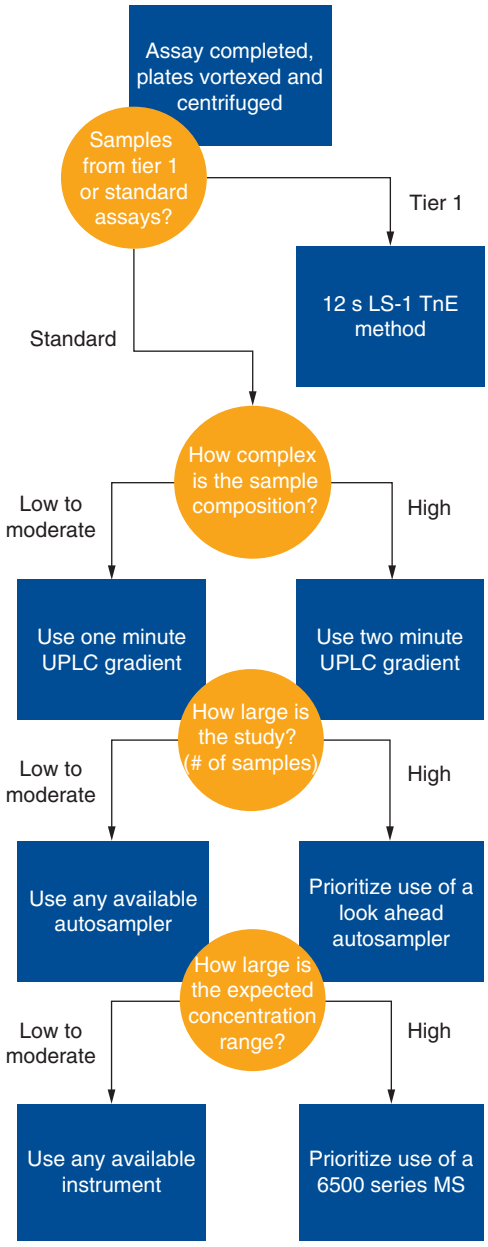

Figure 4. Decision tree used for instrument selection for analysis of tier 1 and standard *in vitro* assays. This process allows for optimal instrument and ultra performance liquid chromatography method selection for each *in vitro* assay type (quality), minimizing turnaround time (speed).
MS: Mass spectrometer; TnE: Trap and elute; UPLC: Ultra performance liquid chromatography.

last of which often require lower limits of quantitation. Additionally, any compound optimization that the software deems unsuccessful (e.g., no peak at the expected mass-to-charge ratio, insufficient signal) in positive mode is automatically tested in negative mode (speed).

At a rate of approximately 1.5 min per compound (~3 min per compound prior to LS-1 use), this process is completed overnight (speed). Over the most recent ~7000 methods developed, the first-pass success rate (no manual intervention) was >97% based on our internal acceptance criteria, which is more stringent than that built into the software (quality). Approximately half of the remaining compounds passed the initial method development procedure, but the highest observed fragment peak height was below 5×10^5 c.p.s., which was determined as a pseudo-minimum acceptable peak intensity based on our specific instrument, the method development sample concentrations and our *in vitro* assay starting concentrations. These compounds are then resubmitted, most commonly either at a different charge state (e.g., compounds with MW >850 Da are set as 2+ as a first intent) or utilizing a higher-concentration stock solution. One point to highlight is that these criteria are regarded as guidelines, and the scientist performing the method development has discretion to accept methods outside the parameters discussed based on previous experience with the chemical space or projects of the failing compounds. It

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should also be noted that methods developed on our Sciex 5500 instruments have been broadly applicable across Sciex 5000 and Sciex 6500 series instruments. Although the source parameters are different for each instrument type (as shown in Table 1), DiscoveryQuant automatically adjusts some MRM parameters from the database that are instrument-dependent. This has allowed a simple drag-and-drop approach for the compound-specific parameters (e.g., collision energy, declustering potential) to be highly successful.

Although most compounds within our organization go through the tier 1 assays and thus the MRM method development process described above, it is preferential to have additional information for compounds being progressed to higher-value assays. Every compound that advances into standard *in vitro* assays has an additional method development step taken using LeadScape® to perform a high-pressure liquid chromatography injection using the generic chromatographic conditions shown in Supplementary Table 2. The signal observed on the column is expected to have a peak area $>5 \times 10^5$ counts, a retention time suggesting sufficient interaction with the column, and reasonable peak shape (quality). As mentioned above, this cutoff is specific to our workflow, and we recommend tailoring this value for each laboratory. If a compound fails due to low response, then a reoptimization of the MRM is attempted using the same steps listed above, as if it had failed during the initial MRM optimization. If the compound yields poor peak shape or retention on the column, basic conditions can be tested (as mentioned in the Methods section), or a note can be added in the database suggesting other conditions based on scientist experience, which helps the assay bioanalyst to troubleshoot their study efficiently (speed). A bonus of these workflows is that the methods can also be applied to *in vivo* studies, reducing the method development burden on scientists involved with those analyses (speed).

Analysis of tier 1 *in vitro* assays

Based on the throughput required for the tier 1 assays, along with the internal expectations regarding data TAT and instrument usage, traditional UPLC–MS/MS was not a viable analysis option, and an alternative solution was required. Given our success with DiscoveryQuant software for standard *in vitro* study submission (detailed below) when coupled to Sciex instrumentation, we looked for instrumentation options that mimicked that process but with faster sample-to-sample analysis speeds. Thus we opted to invest in a flexible 'off-the-shelf' solution via the LS-1 autosampler from Sound Analytics (cost), with the primary plan being to analyze the tier 1 assays using online solid-phase extraction (speed), also known as 'trap and elute' (TnE) in Sound Analytics nomenclature.

The LS-1 also provided additional key benefits over other online solid-phase extraction systems such as the RapidFire system from Agilent: first, seamless integration with Sciex mass spectrometers and data analysis software; second, multiple configurations allowing for MRM method development, TnE analysis, and multiplexing UPLC to be plumbed in parallel; and third, practical engineering of the system, including an open-deck layout and robust sampling system, the combination of which can precisely and rapidly sample from any well across ten plates in any order.

Those three key aspects have each proven vital to the success of the tier 1 assays in our bioanalytical lab. While Sound Analytics are the makers of the LS-1 autosampler, they are also directly involved with Sciex on the development of the DiscoveryQuant software, which facilitated the uptake of the LS-1 sample submission and control software LeadScape®. As previously mentioned, the LeadScape® sample submission process is nearly identical to that of the DiscoveryQuant software our scientists were already using for standard *in vitro* assay submission (speed). Additionally, Sound Analytics specifically designed the software to interface with the Sciex instrument control software (Analyst), alleviating the need for additional instrument control software or drivers, which often lead to the communication issues that we have observed to plague mixed-vendor instruments.

The ability to plumb a single LS-1 autosampler in multiple configurations in parallel has also provided significant benefit (cost). Without having to modify the instrument, we can easily switch between developing MRM methods and analyzing tier 1 assay samples, both of which occur on a weekly basis (cost), and developing multiplexing capabilities for future assays. An example of this currently in development in our lab is an additional high-throughput hepatocyte clearance assay, which will require both the speed of the LS-1 autosampler and the selectivity of chromatography. With minimal effort, we can analyze the tier 1 assay samples to start the week, and then transition into determining the optimal conditions for the upcoming hepatocyte assay testing. Additionally, the option to use the system via multiplexing UPLC provided a viable safety net in the situation where TnE analyses and our tier 1 assays/chemical space had not been compatible.

Lastly, the open-deck architecture of the LS-1 and its sampling system generated flexibility with respect to how our *in vitro* assays and analyses were performed. Rather than having to design the assay to output plates with samples

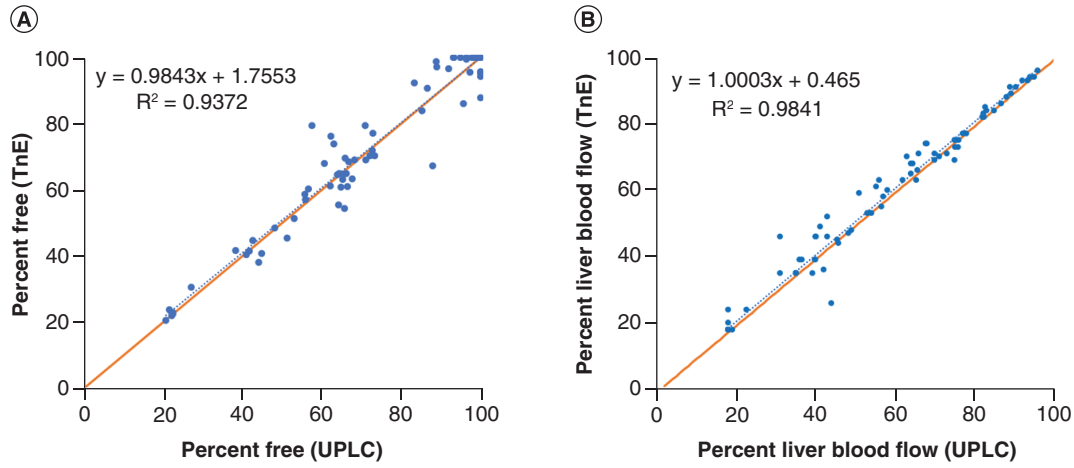

Figure 5. Comparison of analytical approaches for the tier 1 drug metabolism pharmacokinetic microsomal assays.
(A) Correlation plot of % free calculated from the tier 1 microsomal binding assay using a set of commercially available compounds. Samples were analyzed by TnE–MS/MS (20-s method) and then reanalyzed using UPLC–MS/MS (2-min gradient method). **(B)** Correlation plot of % liver blood flow calculated from the tier 1 microsomal clearance assay using a set of commercially available compounds. Samples were analyzed by TnE–MS/MS (20-s method) and then reanalyzed using UPLC–MS/MS (2-min gradient method). Linear data fits depicted with the blue dotted lines. Lines of unity shown as solid orange lines.
TnE: Trap and elute; UPLC: Ultra performance liquid chromatography.

consolidated based on compound, or having an autosampler slowed by the need to shuttle back and forth between open and closed drawers, the LS-1 is able to quickly sample between plates because they are all accessible at the same time. This ultimately unlocks the ability of the LS-1 to provide a rapid analysis approach, and in our hands allows us to inject a sample every 12 s (speed). Furthermore, at that injection-to-injection rate, the needle/loop/syringe injection system has been shown to be reproducible from samples that are 80% acetonitrile. This robust hardware – combined with the optimized solvent conditions and column selection shown in the Methods section (which improved analyte retention consistency from highly organic samples) – means there is no need for further dilution of the samples following a simple protein precipitation, reducing complexity and wasted time along the process (speed, cost, quality).

Despite the obvious speed and cost benefits mentioned above, the quality of the outgoing data was also imperative. To evaluate the potential for TnE–MS/MS analysis to provide reliable data for our early efficacious dose predictions, microsomal binding and microsomal clearance assays were analyzed via both TnE–MS/MS and UPLC–MS/MS. The correlation plots shown in Figure 5A & B represent a group of commercial compounds, with the two analysis approaches yielding values within 25% of each other for 69/70 binding variants and 144/147 clearance variants. Worth noting is that initial assay development and deployment utilized a method set for a 20-s sample-to-sample analysis time. This was later reduced to 12 s to maximize throughput with minimal impact on other performance parameters (e.g., carryover). To ensure continued assay data quality, a set of GSK proprietary compounds were analyzed using each TnE method for both tier 1 assays. The correlation plots generated are shown in Figure 6A & B. Over 96% of the calculated percentage free values were within 25% using the two TnE methods, and over 99% of the calculated liver blood flows within 25%. The internal standard response has also been measured for >500,000 tier 1 samples over the past 2 years, with <0.5% of samples having an internal standard response outside the intra-set internal standard mean ±35%. This low percentage of sample loss due to internal standard failures suggests the slight modifications we have made since the inception of the assays (e.g., daily column backflushing) have improved the analytical variability, and that we could potentially align the cutoff with our standard assays (±25%) without greatly increasing the number of samples lost (quality).

Given the Tecan and LS-1 deck configurations, the assay requirements, a speed of 12 s per sample analysis and the data analysis tools developed (discussed below), we have been able to complete the bioanalytical portion of the two tier 1 assays in a median of 1 day per week. Although this format was successful, concerns over potential downtime (speed, cost if outsourced) and TAT reduction (speed) led to the purchase of a second Tecan Fluent and a second LS-1 autosampler. Based on our experience with the robustness of our first LS-1, the ability to drag and

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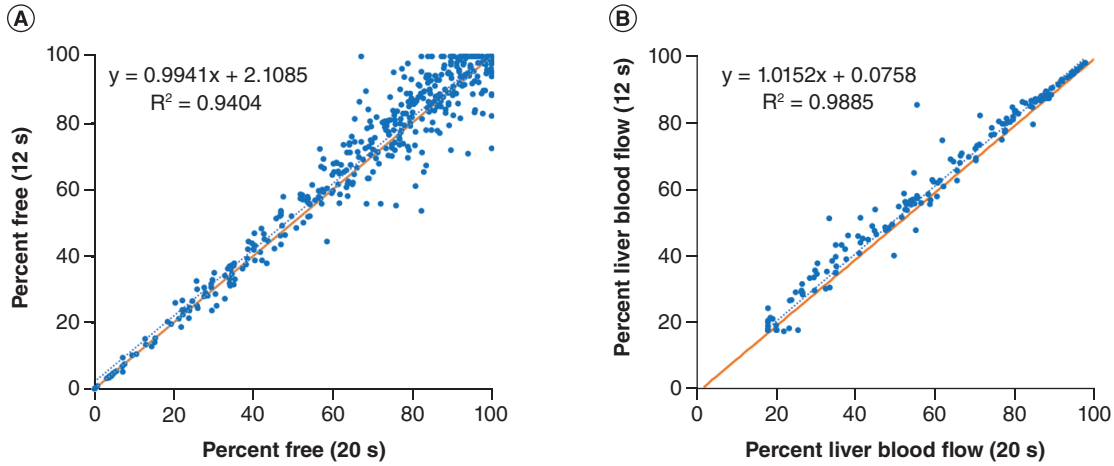

Figure 6. Comparison of trap and elute methods for the analysis of tier 1 drug metabolism pharmacokinetic assays. **(A)** Correlation plot of % free calculated from the tier 1 microsomal binding assay using a set of GSK proprietary compounds. Samples were analyzed using the 20 s/sample TnE–MS/MS method and then reanalyzed using the 12 s/sample TnE–MS/MS method. **(B)** Correlation plot of % liver blood flow calculated from the tier 1 microsomal clearance assay using a set of GSK proprietary compounds. Samples were analyzed using the 20 s/sample TnE–MS/MS method and then reanalyzed using the 12 s/sample TnE–MS/MS method. Linear data fits depicted with the blue dotted lines. Lines of unity shown as solid orange lines.
TnE: Trap and elute.

drop methods to the second instrument, and the high quality of initial tests on the new instrument, it was deemed that sample sets from the assays could be analyzed in parallel based on instrument availability rather than on a matched assay/instrument pair basis (speed), which is aligned with our standard *in vitro* assay bioanalysis workflow.

Analysis of standard *in vitro* assays

Although we no longer use DiscoveryQuant as our primary automated method development software, we continue to use it for submission of all standard *in vitro* assay analyses. DiscoveryQuant provides a simple study submission process predicated around using generic UPLC conditions and imported batch lists. Through collaboration with our *in vitro* colleagues, we developed a system whereby the list of compounds to be analyzed and a batch list containing all the relevant information are automatically generated as tab-delimited text files at study initiation. With those files available, a bioanalyst can query the MRM database for methods, couple them with the assay-appropriate UPLC method, import the batch list and submit a study in less than 5 min (speed, quality).

Despite the ability to rapidly submit studies using DiscoveryQuant and to quickly process and review the data using MultiQuant (discussed below), one key aspect impacting the overall bioanalytical speed remains the actual analysis time. More specifically, the injection-to-injection time was (and still is) dictated by the UPLC method duration and the autosampler's ability to shuttle between samples. Considering this, the assay type being analyzed (quality), the number of samples for each assay (speed) and the MS instrument series capabilities (speed, dictated by the number of reinjections required), we built the decision tree shown in Figure 4, which allows us to support 100% of in-house *in vitro* assays with three full-time employees and two student interns (cost).

In summary, we assign every assay a low/moderate/high score for each of the following three aspects: intra-set analyte concentration range, number of samples for analysis, and complexity (ability for metabolites to be formed in the assay and the relative protein concentration in the sample matrix). These three scores define the ideal analytical details for each study type with respect to the mass spectrometer used, the preferred autosampler and the gradient applied. To elaborate further, assays in which there is a greater range in sample-to-sample analyte concentration are preferentially analyzed on our Sciex 6500 instruments (vs our Sciex 5000 instruments), which takes advantage of the improved sensitivity and linear dynamic range of the newer mass spectrometers. Next, instruments with autosamplers that can more rapidly transition between injections are selected for assays with higher numbers of samples. Specifically, in our laboratory those autosamplers are PAL3 RSI systems with the 'look-ahead' functionality enabled (~30 s savings per sample vs our other autosamplers). Lastly, assays with low and moderate complexity (e.g., MDCK, protein binding) utilize the 1-min UPLC gradient shown in Supplementary Table 3, whereas more

complex assays (e.g., hepatocyte clearance) use the 2-min gradient. This gradient/column aspect is deprioritized because each instrument in our lab is capable of either set of UPLC conditions without the need for scientist intervention.

Although our current instrument configuration does not allow for complete compliance within that instrument selection hierarchy, the framework has helped us analyze 100% of in-house standard *in vitro* assays with a median bioanalytical TAT of 2 days using three LC–MS/MS instruments. Additionally, as ongoing automation efforts are likely to increase MDCK assay throughput, we are currently evaluating the potential for the MDCK assay to be analyzed using a LS-1-Sciex 5500 system like the tier 1 assays discussed above. Given the low complexity, high sample numbers and wide dynamic range requirements for the assay, we feel the TnE–MS/MS approach will be suitable, maintaining data quality, providing the rapid analysis times needed to maintain or improve our TAT, and minimizing free time on our valuable LS-1 MS/MS system (quality, speed, cost).

MultiQuant for high-throughput data analysis

The last step in our bioanalytical workflow is the relative quantitation of each of our samples. For standard *in vitro* assays this can include upwards of 75 compounds, with a range from <20 to >1000 samples per study. Tier 1 assays are run using hundreds of compounds per week and regularly include >5000 samples per week. Given those demands, a traditional Sciex Analyst workflow would not suffice. Thus we evaluated alternative solutions previously discussed in the literature, focusing on 'Sciex-friendly' data-processing software, including MultiQuant, DiscoveryQuant and LeadScape® [35]. Based on our experiences, the Sciex MultiQuant workflow had the simplest and most intuitive workstream, particularly for large numbers of compounds. Additionally, only MultiQuant features the ability to 'query' data using highly customizable Excel-based logic and calculations. Although this capability is not well discussed in the literature to the best of our knowledge [36], it has been instrumental in speeding up our data analysis process, and we highly recommend its use. By highlighting data that fall outside of user-defined acceptable ranges (speed, quality), MultiQuant queries facilitate standard assay workup. This allows for rapid acknowledgment and decisions around troublesome compounds, sample sets or injections and a review-by-exception workflow for the tier 1 assays. Rather than have a scientist look at every sample, they can focus only on the samples that fail our criteria, deciding whether exclusion of the sample is preferred or if some corrective action can be taken within MultiQuant (tier 1 assay samples are not reinjected). This type of tier 1 assay data analysis results in data review that typically takes less than 2 h to cover the entire 5000+ samples (speed).

In our specific scenario, we utilize the query function to prescreen data for the key quality parameters of intra-set internal standard variation, signal-to-background, analyte/internal standard peak height, and peak integration quality. In addition to changing the font color within MultiQuant, the data that fail can be automatically set as unreportable with a query comment generated detailing why the sample failed, so that they are not inadvertently used in downstream calculations (quality). Though this type of data scrubbing can feasibly be done on the assay analysis platform (e.g., Microsoft Excel, Signals VitroVivo), performing it via the query was found to be simpler to implement and kept the decisions within the bioanalyst's control. How these cutoffs are determined, or what level of quality is expected from the data, will be different based on workflows and risk profile, but examples of our expectations are provided below.

For assays analyzed via UPLC–MS/MS, we define the acceptable internal standard range as the intra-set (each set being the samples from a single compound in a single matrix) internal standard mean peak area ±25%. For tier 1 assays, this window was widened to 35% to reduce sample attrition. Peak height is set to highlight samples that are likely to be outside the linear dynamic range of the instrument used (e.g., peak height $>3 \times 10^6$ for a Sciex 5000; peak height $>4 \times 10^7$ for a Sciex 6500). Signal-to-background, defined as the analyte peak area of a sample divided by the analyte peak area for the corresponding matrix blank at the same retention time, is expected to be >5 for standard assays and >3 for tier 1 assays. Lastly, peak quality is a parameter defined within MultiQuant that attempts to correlate how well a peak is integrated, with a value between 0 and 1; our typical initial screening value is set at 0.9.

Within our workflow, failures due to internal standard response, peak height and signal-to-background are automatically set to not be reported. Our experience with peak quality is that low signal or tailing peaks can give failures where the integration quality is fit for purpose, and thus our peak quality flag is used only to highlight samples that need extra review rather than automatically nullifying the data. Additionally, we allow scientific discretion outside of these predefined acceptance criteria but require that the rationale be recorded in the assay electronic notebook.

One current downside of MultiQuant pertains to its use for the workup of tier 1 assay samples. The speed of the LS-1 requires that a single .wiff data file be used for the multiple injections within each sample set. Consequentially, metadata of each sample (e.g., sample name, sample type) are lost when the data are processed. We currently utilize Excel-based macros to rename samples post-analysis, and we hope to incorporate this function into a custom bioanalytical laboratory information management system in the future. This issue could also be alleviated by using the data analysis portion of the LeadScape® software package; however, for our use the benefits of MultiQuant described above outweigh this negative. We are also aware that the Sciex OS platform utilizes a data-splitting algorithm to solve a similar issue for Echo-MS data [37]; however, this platform was not widely available during the development of our workflow. Additionally, the Sciex OS algorithm requires specific 'extra' injections at the start and finish of each sample set. This increases the analysis time by roughly 1 min per sample set, which would result in a 100% increase in analysis time for our microsomal binding tier 1 assay, for example. Lastly, Sciex OS does not have the ability to create easily customizable and reusable queries. Based on these aspects, at this time we have opted not to investigate Sciex OS for the data analysis portion of our LS-1-based assays.

Conclusion

To match the increased *in vitro* DMPK assay throughput established at GSK in the past 3+ years, our bioanalytical group has established a workflow built around software and hardware solutions from Sciex and Sound Analytics. Automated MRM method development using LeadScape® sets the foundation for analyses to be completed via UPLC–MS/MS or TnE–MS/MS, with the TnE studies performed using LS-1 autosamplers. The generation of study batch lists has been set to occur automatically at study creation, enabling the use of DiscoveryQuant or LeadScape® (LS-1-based studies) for rapid study submission immediately upon sample availability. Lastly, MultiQuant is used to handle these large datasets via the use of the built-in query functionality coupled to custom Excel-based logic that screens the data based on key quality parameters. These improvements have allowed for bioanalysis of 100% of in-house *in vitro* assays over the past 2 years, with a median bioanalytical TAT of between one and two business days.

Future perspective

Regardless of the *in vitro* study type (assuming compound throughput is high enough to require assay automation), the rate-limiting step of the bioanalytical portion is the physical sample analysis and, more specifically, the time needed for the UPLC or TnE gradients. For many assay types, these are required to improve limits of detection, separate the analyte of interest from matrix components and/or metabolites, or minimize the instrument-cleaning requirements. For assays where these limitations are minimal and analysis speed is a priority, the Echo MS system from Sciex holds a great deal of potential. Coupling acoustic ejection and an open port interface, the system can sample as fast as three wells per second from either 384- or 1536-well plates, with minimal matrix effects reported based on sample dilution within the carrier solvent [5,24]. Additionally, Sciex has coupled with Beckman Coulter Life Sciences as its preferred automation partner to enable the use of Echo MS as a walk-away analysis platform. However, beyond the hardware capabilities of the system, one critical evaluation point is how the system control software can support a given study workflow. Although Sciex continues to develop the software platform supporting the Echo MS, at this time the system requirements for studies with large numbers of analytes within a given run sacrifice some (if not all) of the speed advantages.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2022-0207

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Executive summary

Introduction

- The mantras of 'fail early' and 'fail fast' within drug discovery organizations have led to an increase in the implementation of moderate- to high-throughput *in vitro* drug metabolism pharmacokinetic (DMPK) assays, which are used as part of rapid design/make/test/learn cycles.
- To deliver the sample analysis required by high-throughput *in vitro* DMPK assays, we assessed the hardware and software available across the industry, focusing on aspects that would provide suitable speed and quality at an acceptable cost.

LeadScape® for automated method development

- Many *in vitro* DMPK assays require that the relative amount of each unique compound be measured, necessitating an automated method development platform.
- LeadScape® and DiscoveryQuant™ software packages both provide straightforward and rapid automated approaches to MS/MS method development and evaluation of generic chromatographic conditions.

Analysis of tier 1 assays

- Our weekly tier 1 DMPK assays generate thousands of samples for analysis, a volume that cannot reasonably be handled using traditional ultra performance liquid chromatography (UPLC)–MS/MS approaches.
- We have implemented highly flexible and robust LS-1 autosamplers from Sound Analytics, each of which are used in trap-and-elute mode for tier 1 assays, with a sample-to-sample time of 12 s.

Analysis of standard *in vitro* assays

- DiscoveryQuant provides an efficient study submission process predicated around using generic UPLC conditions and imported batch lists.
- We utilize generic 1- and 2-min UPLC gradients dependent on the sample complexity, and determine the analysis instrument based on intra-set analyte concentration range and the number of samples for analysis.

MultiQuant™ for high-throughput data analysis

- MultiQuant provides a simple and intuitive workflow for the analysis of large datasets stemming from high numbers of compounds.
- User-built Excel-based queries can be used within MultiQuant to highlight data based on key performance standards, facilitating sample troubleshooting and enabling a review-by-exception workflow when required.

Future perspective

- Improvements in bioanalytical turnaround time will likely hinge on the ability to analyze each sample more rapidly, which has historically been defined by the timescale of the sample clean-up step (e.g., UPLC, on-line solid phase extraction).
- The Echo-MS system from Sciex couples acoustic ejection with an open port interface, allowing for analysis rates of as fast as three samples per second.
- Samples are diluted within the open port interface, which has been reported to reduce the matrix effects traditionally minimized via slow sample clean-up steps.
- Uptake of the Echo-MS system will be highly dependent on the ability of acquisition and data analysis software to match the capabilities of the hardware.

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