True High Throughput Bioanalysis Using the Echo® MS System

Rapid Sample Analysis Using AEMS Technology Improves Bioanalytical Study Turnaround

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Analysis of biological samples to assess the pharmacokinetic (PK) properties of drug candidates is a cornerstone assay that is routinely performed in virtually all pharmaceutical companies. Despite the importance of PK information in drug discovery and development, bioanalytical data is time consuming data to produce. Many individual time points must be analyzed, often from multiple subjects, to generate data for a single compound. When considering the time required to prepare samples, even just using a simple protein precipitation, in addition to 1 to 2 minute individual sample analysis times in the quickest cases, data is generally not available until the day after the bioanalyst begins work on a study.

The SCIEX Echo® MS System, which uses Acoustic Droplet Ejection technology and an Open Port Interface (OPI) coupled to the high-sensitivity SCIEX Triple Quad™ 6500+ Mass Spectrometer, is capable of dramatically reducing analysis time, as well as reducing the need for sample preparation.[1] This allows PK study data to be available the day samples are prepared, giving researchers faster access to this important data (Figure 1).

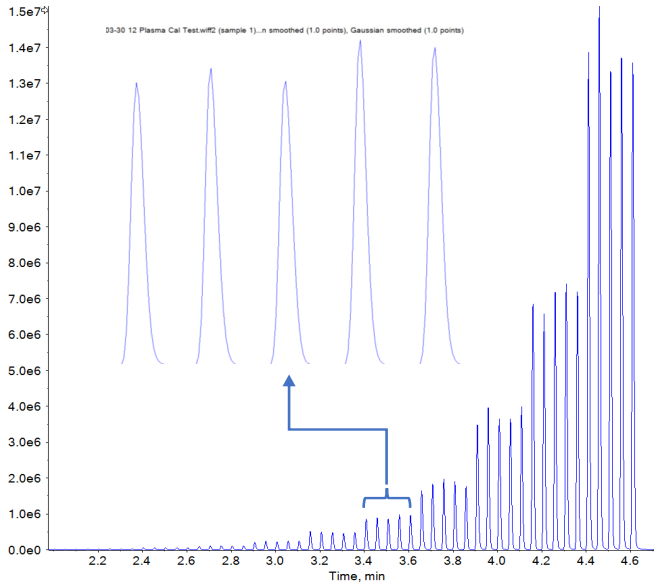

Figure 1. Complete pharmacokinetic study in plasma in under 5 minutes. A calibration curve of fentanyl in untreated plasma was generated across 16 different concentrations with 5x replicates at each point in under 5 minutes.

Selection of the most appropriate sample preparation method is always a balance of required sensitivity, necessary throughput and the cost of consumables like SPE cartridges. For bioanalytical assays where sensitivity requirements are easily met, faster analysis and simpler sample prep lead to a more efficient overall process. Here, very simple sample preparation techniques were explored in combination with the SCIEX Echo® MS System for rapid generation of routine bioanalysis studies.

Key features of Echo MS System for routine bioanalysis

- Integral to the Echo MS System is the high-sensitivity SCIEX Triple Quad 6500+ System for meeting sensitivity requirements
- Good quantification limits were achieved with all three simple sample preparation techniques that were explored, meeting the needs of many routine bioanalysis studies for throughput and cost
- Very high reproducibility of analysis obtained even out of untreated plasma
- Exceptional speed and low carryover of Echo MS System combined with simplified sample preparation demonstrated here make this an efficient and fit-for-purpose bioanalysis platform

Methods

Sample preparation: Fentanyl (Cerilliant) working standards were prepared by serial dilution in 10% v/v methanol in water from 65.536 µg/mL to 0.002 µg/mL in 1:1 increments. Norfentanyl (Cerilliant) was used as an internal standard, and was diluted to 200 ng/mL in 10% methanol in water.

10 µL of working standard and 10 µL of internal standard was added to 200 µL of K_2EDTA Sprague-Dawley rat plasma (Bioreclamation IVT). Identical calibrations were also prepared in 0.1% and 0.01% by volume PEG400 (Sigma) in rat plasma.

Protein precipitation: 100 µL of prepared plasma was transferred to a conical bottom 96 well plate. 100 µL of methanol was added to each standard, the plate was covered and mixed gently on an orbital plate shaker for 5 mins. The plate was centrifuged at 4000 rpm for 10 mins to settle the precipitate. 60 µL of supernatant was transferred to an Echo 384 well plate (Beckman Life Sciences 384PP 2.0 Microplate) for analysis.

1:1 dilution: 30 µL of the prepared plasma samples was transferred to an Echo 384-well plate. 30 µL of water was added to the samples, and the plate was gently mixed on an orbital shaker for 5 mins.

Untreated plasma: 60 µL of the prepared plasma samples were transferred to Echo Qualified 384-Well Polypropylene Microplate 2.0 (384PP 2.0).

Prior to analysis, the final sample plates were centrifuged at 3000 rpm to remove any bubbles in the wells, and then briefly mixed on an orbital shaker to ensure formation of a stable meniscus in each sample well.

Mass spectrometry: MRM data was collected in positive polarity using the SCIEX Triple Quad 6500+ System using the parameters outlined in Table 1. Key ion source parameters are outlined in Table 2. Note that the value for GS1 value is 90 for this assay, this value is required to be higher than usual because the GS 1, or nebulizing gas flow, is what provides the aspirating force that pulls the carrier solvent from the OPI into the ion source.

Table 1. MRM parameters.

Compound	Q1	Q3	Dwell	DP	EP	CE	CXP
Fentanyl	*337.2*	*188.1*	*45*	*70*	*10*	*30*	*11*
Norfentanyl	*233.0*	*84.1*	*45*	*55*	*10*	*24*	*5*

Table 2. Ion source settings.

Parameter	Setting
GS 1	*90*
GS 2	*70*
CUR	*30*
CAD	*12*
Temp	*350*
IS	*5000*

Acoustic droplet ejection methods: Acoustic droplet ejection methods, which are analogous to HPLC methods for the Echo MS System, contain relatively few parameters.

Flowrate will generally be based on the viscosity of the carrier solvent , which was pure methanol for this study.

Fluid class will be based on the viscosity of the sample liquid; SP for liquids less viscous than water, AQ for samples in liquids with viscosity equal to or greater than water.

Delay time can be added to an acquisition to allow for ejections of “richer” matrices to come completely back to baseline between sampling. For bioanalytical work, an additional 1000 msec delay time was used to ensure high concentration samples did not elevate the baseline of subsequent low concentration samples. More aqueous or solvent based samples do not generally require this.

Droplet count is the number of droplets pulsed in very short succession as a single ejection and is analogous to an injection volume in HPLC work. Optimal droplet count will depend on the matrix and required sensitivity. Richer matrices can show diminishing returns at higher droplet counts as suppressive effects and peak broadening counteract the greater amount of analyte introduced into the liquid stream. Cleaner matrices allow for often significant increases in sensitivity (up to 20 droplets in some cases). When assays are being developed, running a droplet ladder is helpful to select the optimal droplet count.

Figure 2 shows a droplet ladder experiment run using a standard from the protein precipitation calibration. In Figure 2, a linear signal increase is observed for 1 through 5 droplets, with the response leveling off between 8 and 9 droplets. 8 droplets was chosen for this matrix type.

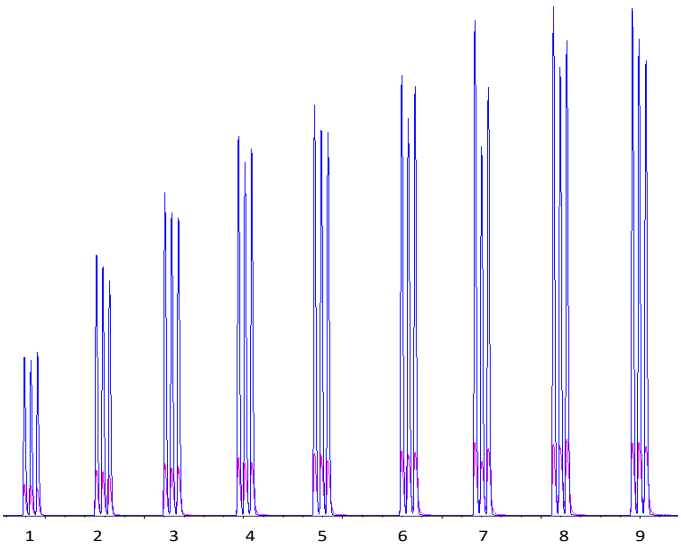

Figure 2. Droplet ladder for the samples prepared by protein precipitation. Performing a droplet ladder experiment during assay development allows the user to balance signal with matrix effects. Here the x axis is droplet count (n=3 replicates per count) for fentanyl in protein precipitated plasma. Eight droplets was the optimal number determined here.

Table 3 below lists the parameters for each acoustic droplet ejection method for the three different sample preparation experiments.

Table 3. Acoustic droplet ejection method parameters.

Parameter	Plasma	1:1 Dilution	PPT
Flowrate	*350*	*350*	*350*
Fluid Class	*AQ*	*AQ*	*SP*
Additional Delay	*1000 ms*	*1000 ms*	*1000 ms*
Droplet Count	*5*	*4*	*8*

Data processing: Once acquisition of a batch is complete, a file splitting algorithm automatically runs which creates individual files within the acquired .wiff2 file that can be processed in SCIEX OS Software Analytics module. In high-throughput environments, where calculations are done by a LIMS or other software, peak areas can automatically be generated if a Processing Method is specified in the batch. For this example, the data is processed the same as standard bioanalytical data in Analytics.

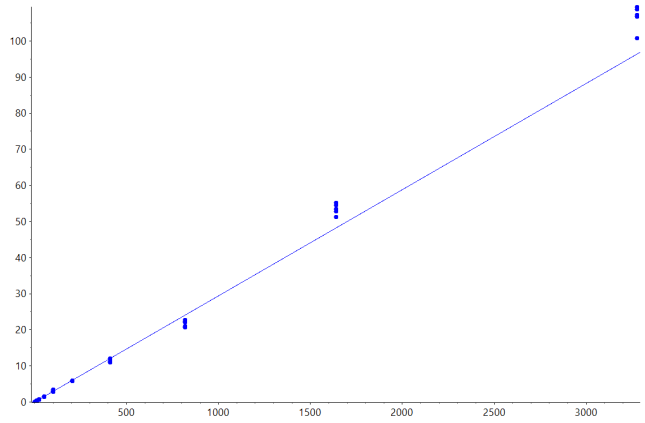

Figure 3. Example calibration curve for fentanyl in untreated plasma. Concentration curve from X to Y ng/mL of fentanyl in untreated plasma was generated and run with n=5 ejections each concentration level.

Sensitivity achieved with different sample preparation methods

Three different sample preparation methods were explored, to characterize the impact of matrix complexity on the quality of data generated. The concentration curves for fentanyl were generated in each and the lower limits of quantification (LLOQ), linearity and reproducibility were determined. Figure 3 shows an example curve for fentanyl in untreated plasma. Blanks were performed before the lowest concentrations ejections to confirm the LLOQs (Figure 4). Overall, the best performance was observed for the data obtained from the untreated plasma.

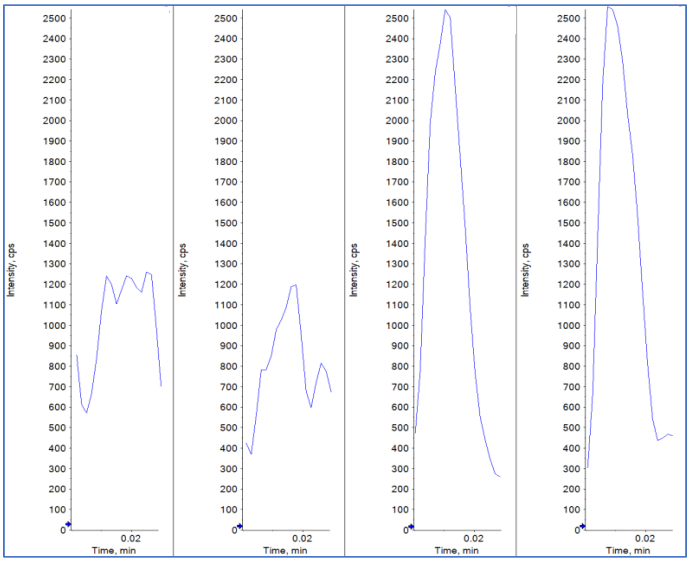

Figure 4. Signal for fentanyl at lower limit of quantification. Blank samples followed by standard ejections of the fentanyl in untreated plasma at a concentration of 0.2 ng/mL, demonstrating good signal at the LLOQ.

Table 4. Reproducibility of the calibration curves in each sample preparation strategy. The %CV for n=5 reps for each calibration level was calculated, with the majority being below 5% across the concentrations.

Std Conc.	Plasma	1:1 Plasma: Water	Protein Precipitation
0.1	*BLOQ*	*BLOQ*	*BLOQ*
0.2	*9.4*	*BLOQ*	*BLOG*
0.4	*7.6*	*10.6*	*9.5*
0.8	*5.5*	*	*6.4*
1.6	*10.3*	*5.6*	*7.6*
3.2	*3.5*	*8.1*	*6.9*
6.4	*3.9*	*3.9*	*4.7*
12.8	*3.8*	*8.8*	*1.9*
25.6	*6.8*	*4.2*	*4.8*
51.2	*2.4*	*3.9*	*6.6*
102.4	*7.7*	*3.4*	*2.9*
204.8	*1.8*	*7.2*	*6.1*
409.6	*4.2*	*3.9*	*4.9*
819.2	*3.9*	*3.7*	*6.9*
1638.4	*2.8*	*4.4*	*5.2*
3276.8	*3.2*	*3.2*	*5.9*

** Standard was lost during transfer*
BLOQ Standard was below the limit of quantitation

Average accuracy for all replicates across the concentration curves was between 85 and 115% of nominal for all preparation methods. The reproducibility at all concentration levels for each sample preparation technique is listed in Table 4. It is worth noting that for all three preparations, there was not a significant difference in either assay sensitivity or variability.

Evaluation of suppression effects

In addition to sensitivity, reproducibility and linearity, the effect of potential matrix suppression was also investigated by analyzing standards prepared in plasma fortified with PEG400, which were treated as quality controls when the data was processed. PEG400 is a common formulation agent used in pharmaceutical research that can have significant suppressive effects in electrospray mass spectrometry. Stable labeled internal standards generally compensate for ionization suppression, as long as the analyte is present at a level high enough above the lower limit of quantification to still be measurable. In research PK work, labeled internal standards are not always available, so chemically similar compounds are often selected for use. Even when chemically similar, ionization suppression does not always effect analytes and internal standards equally, especially if they are chromatographically resolved. In this case norfentanyl, which is a metabolite of fentanyl, was used as an IS and there is no chromatography to consider. The effects of different levels of PEG400 in untreated plasma can be seen in Table 5 at the 102.4 ng/mL level as an example. The deviation from nominal at other concentrations was very similar to the 102.4 level for the two PEG400 levels.

Table 5. Matrix effects observed for different PEG400 concentrations. Standards (top row) and QC Samples prepared at the 102.4 ng/ml level. Values are of n=5.

Matrix	Peak Area %cv	Average Calculated Concentration	Average Accuracy
Plasma, no PEG400	*7.7*	*104.8*	*102.4%*
Plasma w/ 0.01% PEG400 by volume	*5.3*	*99.4*	*97.1%*
Plasma w/ 0.10% PEG400 by volume	*3.2*	*76.7*	*74.9%*

Conclusions

The goal of this study was to assess the feasibility of the Echo MS System for routine, early stage bioanalytical quantification. Three plasma sample preparation techniques were explored to evaluate the effect minimal sample preparation would have on the quality of data produced. The approaches used in this project show minimal difference for dilution, basic protein precipitation and simply analyzing plasma directly without any treatment at all.

For routine analysis, where the assay sensitivity requirements demonstrated here are sufficient, the speed and simplicity of analysis using the Echo MS System offers an attractive approach for high turnaround bioanalytical laboratories.

In future work, the use of more thorough preparation techniques such as SPE, will be explored for the impact on sensitivity. In addition, the use of labeled internal standards will also be explored to further improve the data quality.

References

1. Rapid MS/MS analysis with Acoustic Ejection Mass Spectrometry (AEMS) - Using the SCIEX Echo® MS System to break bottlenecks in quantitative mass spectrometry throughput. SCIEX technical note RUO-MKT-02-11385-A.

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