# True high throughput analysis of a transporter activity assay

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## ABSTRACT

Analysis of the large number of samples generated by an in-vitro transporter activity assay is generally done by LC-MS/MS which creates a limitation on the number of compounds that can screened for this important ADME property. Here we show transporter activity and membrane permeability data for eleven model small molecule compounds, which translated to over one thousand samples and standards to be analyzed. This dataset was collected over the course of a single workday using acoustic ejection mass spectrometry on an Echo® MS system.

## INTRODUCTION

A key property used to predict *in-vivo* exposure of a drug candidate is its ability to permeate a cell membrane as well as it's potential to act as a substrate of transmembrane transporter proteins. One of the most widely distributed membrane proteins responsible for the transport of xenobiotics across cellular membranes is generically called permeability glycoprotein 1 (Pgp). The LLC-PK cell line can be engineered to overexpress variants of Pgp (notably murine derived Mdr1a and human HMDR), and membranes grown from enriched and non-enriched sources are used to assess a drug candidate's potential as a substrate for this important transporter protein. Evaluation of a drug candidate's permeability and transporter activity is a complex process that requires analysis of many samples to assess a single compound's activity. Sample wells are prepared in replicate to account for an individual membranes that do not grow as planned. Multiple timepoints are taken for each well, apical to basolateral and basolateral to apical (A to B and B to A) permeability is measured, and overexpressed Pgp membranes are compared to natively expressing membranes. This can easily result in sixty or more individual samples that need to be collected, analyzed and quantitated by LC-MS/MS to assess the permeability and transporter activity of a single compound. In this study, the Echo® MS system was used to analyze samples generated from a multi-compound transporter study, simultaneously acquiring data for up to four compounds per 384 well plate, corresponding to as many as 300 total ejections including samples, standards and blanks, in a total time of about 13 mins per plate. An analysis time of 2 seconds per sample was used to ensure adequate baseline resolution per sample. When compared to even a short analysis time of 3 min per sample by LC-MS/MS, the time savings works out to approximately 15 hours per plate using the Echo MS system as an analytical platform.

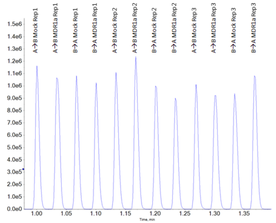
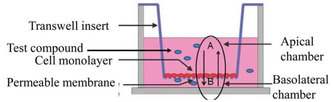



**Figure 1.** Time 0 samples of lamotrigine. Three laboratory replicates of A→B and B→A for both MDR1a upregulated and native LLC-PK membranes. 12 samples analyzed in 24 seconds.

**SCIEX Echo® MS system**

## MATERIALS AND METHODS

**Sample preparation:**
Samples were generously provided by a collaborator in 96 well plate format. The following is a brief description of their generation. LLC-PK (porcine kidney monolayer) cells containing either only natively expressed transporter proteins, or engineered to highly express Mdr1a or HMDR, were grown in Millipore 24 transwell plates,




with a membrane surface area of 0.7 $cm^2$. Apical well volumes of 400 mL and basolateral volumes of 800 mL were used, with an initial donor side concentration of 2 mM of the test compound. There were two different experimental designs; one was the comparison of native to Mdr1a, with time points taken at 30, 60 and 90 minutes, and a second comparing native, Mdr1a and HMDR enriched membranes, with timepoints taken at 15, 30, 45 and 60 minutes. All compounds for all membrane types were run apical to basolateral (A→B) and basolateral to apical (B→A), with each being run in triplicate. Calibration curves for each compound were provided with nominal concentrations of 2, 1, 0.2, 0.02 and 0.002 mM each. Dextromethorphan was added as an internal standard to all samples and standards post collection at an unstated concentration.
96 well plates were arranged in a manner so that no more than 4 analytes were present in a plate when they were transferred to 384 well plates, so each 384 well plate could be analyzed with a single method in a single batch. Prior to analysis, 50 mL aliquots were transferred to Echo qualified 384 well plates using a 12-channel pipette. Plates are available from Beckman Coulter Life Sciences. The plates were then centrifuged at 3000 rpm for 5 mins to remove any bubbles and the shaken on a plate mixer at 1350 rpm for 1 min to ensure a stable meniscus was formed on the surface of the samples. Plates were stored at room temp with an adhesive cover until analysis on the Echo MS system.

**Acoustic ejection parameters:**
One acoustic ejection method and carrier solvent was used for all analysis. A single 2.5 nL droplet sample size was used with the AQ fluid class. Pure methanol was the carrier solvent run at 420 mL per minute. Sample ejections were run at one sample every two seconds to ensure adequate time for baseline resolution between peaks.

**Table 1. Source settings**

| Parameter | Value |
|---|---|
| *IS Voltage* | *5000* |
| *Curtain gas* | *25* |
| *GS 1* | *90* |
| *GS 2* | *70* |
| *Temp* | *350* |

**MS/MS conditions;**
A total of 11 compounds were analyzed for this study, with 8 of them being prepared and analyzed using both of the experimental designs discussed in the sample prep section. Common source settings were used for all methods, listed in Table 1.

**Data processing:**
All sample data was integrated and quantified in Analytics module in SCIEX OS software, using the ~~using the~~ summation algorithm. The summation algorithm is most appropriate for Echo MS system data because it does not rely on identifying local minimums around a retention time to establish start and stop points for peak integration. The summation algorithm integrates peak areas as all area above a baseline, between method specific start and stop times. This approach is very consistent, and once the values are appropriately set, virtually no user modification of peak integration is required, which is a distinct advantage when handling the sample volumes produced by Echo MS system.
Permeability values were calculated in using Microsoft Excel. While it's beyond the scope of this document to describe in depth the calculations involved in describing permeability and transporter activity, what was used for this study is briefly described below.

$$Papp = \frac{dQ/dt}{A * C}$$

Where $P_{app}$ is the apparent permeability, $^{dQ}/_{dt}$ is the rate of change in concentration of the analyte of interest in the acceptor well, *A* is the surface are of the membrane and *C* is the initial concentration of the donor well. These values are calculated using average values for each timepoint (n=3) for each time course (i.e. A→B, Mdr1a enriched membrane).

$$Er = \frac{Papp\ B \rightarrow A}{Papp\ A \rightarrow B}$$

Using the A→B and B→A series for each cell line, an efflux ratio was calculated

$$N_{er} = \frac{Er_{MOCK}}{Er_{Mdr1a}}$$

To further describe the activity of the enriched transporters over any natively occurring active transport, a net efflux ratio is calculated.

$$100 * \frac{(Conc\ T_{last} * Vol)_{Acceptor\ well} + (Conc\ T_{last} * Vol)_{Donor\ well}}{(Conc\ T_0 * Vol)_{Donor\ well}}$$

As a check on the possibility of any nonspecific binding or instability of the analytes of interest, an overall recovery was calculated.

## RESULTS AND DISCUSSION

The list of compounds analyzed in this study, with their limits of quantitation, *Er* and *Ner* and %recovery values are shown in Table 2. It should be noted that salicylic acid and digoxin, which happened to be the two compounds to ionize in negative polarity, showed poor sensitivity in this study. That combined with their known low permeabilities, made it impossible to calculate efflux ratios. With further method development, improved limits of quantitation could certainly be reached. Figure 2 shows an example of the raw data acquired by the Echo MS system for the first combined sample plate, monitoring four analytes and an internal standard.

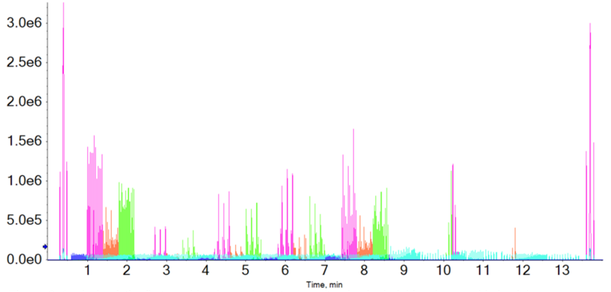



**Figure 2**. Analysis of the first combined sample plate. Lamotrogine in dark blue, loperamide in pink, domperidone in orange, verapamil in green and the internal standard dextromethorphan in light blue. Note the "marker well" signals at the start and end of the batch.

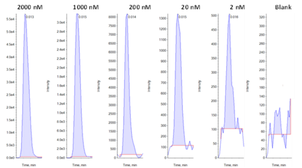



**Figure 3**. Lamotrigine calibration standards and blank.

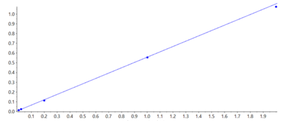



**Figure 4.** Lamotrigine calibration curve.

**Table 2**. Results for compounds in non enriched (MOCK) membrane permeability compared with Mdr1a enriched membranes

| Compound | LLOQ (nM) | %Rec A→B MOCK | %Rec B→A MOCK | $E_R$ MOCK | %Rec A→ B Mdr1a | %Rec B→A Mdr1a | $E_R$ Mdr1a | $N_{er}$ |
|---|---|---|---|---|---|---|---|---|
| *Lamotrigine* | 2 | 92.7 | 90.2 | *2.5* | *88.5* | *93.5* | *2.3* | *0.9* |
| *Loperamide* | 2 | 78.9 | *83.1* | *1.9* | *102.7* | *89.5* | *42.3* | *21.8* |
| *Verapamil* | 2 | 92.2 | *109.0* | *2.9* | *99.4* | *104.5* | *11.5* | *4.0* |
| *Domperidone* | 20 | 81.1 | *112.9* | *8.1* | *83.5* | *96.4* | *376.3* | *46.3* |
| *Quinidine* | 200 | 91.5 | *94.1* | *3.7* | *110.0* | *92.3* | * | * |
| *Risperidone* | 2 | 101.8 | *98.2* | *2.0* | *109.7* | *100.3* | *10.0* | *5.1* |
| *Cetirizine* | 2 | 89.1 | *82.6* | *2.1* | *80.5* | *120.2* | *36.9* | *17.4* |
| *Metoclopramide* | 2 | 88.6 | *109.0* | *4.0* | *96.0* | *128.8* | *10.8* | *2.7* |
| *Loratadine* | 2 | 65.8 | *90.7* | *2.7* | *80.6* | *99.7* | *2.8* | *1.0* |
| *Salicylic acid* | *200* | *95.2* | *96.8* | * | *97.4* | *94.9* | * | * |
| *Digoxin* | *200* | *84.1* | *101.1* | * | *104.5* | *110.5* | * | * |

*The combination of high LLOQ and low apparent permeability did not allow for calculation of these values

## CONCLUSIONS

Overall, the Echo MS system has been shown to be a viable platform for the high throughput analysis of transporter activity samples. In the cases described where sensitivity was not adequate to calculate $E_R$ values, further work can be done to optimize sensitivity – specifically for negatively ionizing compounds. Due to the diverse nature of compounds an ADME group is likely to work with, refinement would best be approached in a generic way in order to preserve the platform nature of the assay. Simply increasing the droplet count from 1 to 10 could have a net effect of a ten fold increase in assay sensitivity[1]. Alternately, for negative ionizing compounds a more appropriate carrier solvent, perhaps with a modifier, could be employed.

Despite the opportunities for sensitivity improvement for some compounds, the time savings gained using the Echo MS system over conventional LC based analysis is significant. Compared with even short LC analysis times of single-digit minutes per sample, the Echo MS system time of 2 seconds per sample allows entire assays to analyzed in minutes as opposed to with overnight runs.

## REFERENCES

1. Rapid MS/MS analysis with acoustic ejection mass spectrometry. *SCIEX technical note, RUO-MKT-02-11385-A.*

## TRADEMARKS/LICENSING