Fully-integrated, high-throughput, dual-stream microflow LC-MS/MS for *in vitro* screening bioanalysis

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Introduction

Biopharmaceutical screening groups positioned in early discovery simultaneously optimize quality, throughput, complexity, and cost of operations. LC-MS/MS methods have been developed that deliver very good throughput (~10s/sample) for analysis of high-throughput *in vitro* assay samples at cost of resource consumption, reproducibility and/or sensitivity. Microflow liquid chromatography (µfLC-MS/MS) can deliver enhanced sensitivity at low flow rates (1-50uL/min), however, utility in supporting *in vitro* screening has not been widely described. Herein we describe integrated, high-throughput, dual-stream µfLC-MS/MS analysis of an optimized *in vitro* human hepatocyte (HHEP) clearance assay. The resultant workflow delivers higher data quality and 25% increase in effective throughput (7.5s/endpoint vs 10s/endpoint) while using 30-fold less LC solvent and can be readily deployed in an enterprise screening environment.

Materials and Methods

- Hepatic drug-metabolizing enzyme substrates midazolam, naloxone, propranolol, triazolam, carbazeran and verapamil were obtained from Sigma.
- Biomek i7 automated liquid handler was used for *in vitro* assay conduct and post-assay sample consolidation (Beckman, Indianapolis IN).
- 6500 Triple Quadrupole Mass Spectrometer was controlled by Analyst 1.7 HF3 software (SCIEX, Framingham MA) and paired with 2x Agilent 1290 Infinity binary pumps.
- LS-I sample-delivery system, controlled by LeadScape software (Sound Analytics, Niantic CT) was equipped with 50µ ID Thermo NanoViper tubing. Microflow separation was performed with 25x0.3mm Symmetry C18 columns (Waters, Milford, MA).

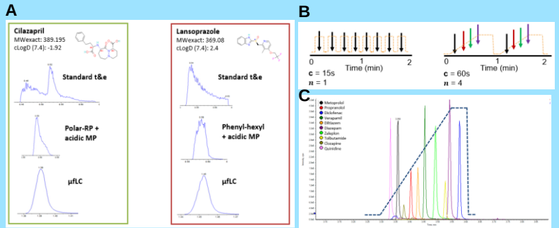


Figure 1a-c: Implementation of µfLC-MS/MS for screening bioanalysis yields significant enhancements to efficiency by way of eliminating laborious bioanalytical remediation and resource spend (A). Legacy high-throughput methods optimize sampling frequency (left) to maximize data collection rate. chromatographic separation afforded by µfLC (right) enables analysis of multiple endpoints per sample (B). Single-stream µfLC method delivers baseline separation of 10 analytes at a rate of 60s/injection (C).

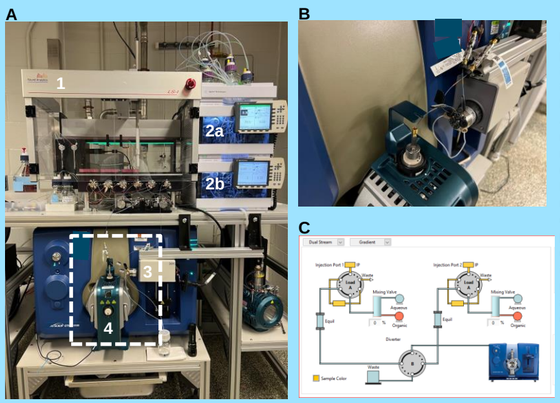


Figure 2a-c: A prototype dual-stream µfLC-MS/MS system was developed to further enhance bioanalytical throughput. The system is comprised of LS-I sample delivery platform (1), dual Agilent 1290 Infinity pumps (JetWeaver mixers bypassed, 2a, 2b), externally-mounted 10-port UHPLC diverter valve (3), and (4), Optiflow MS/MS source (A). Inset from (A) depicts two identical microflow columns interfacing with external diverter valve in very close proximity to MS/MS source-plumbing is comprised of NanoViper 50µ x 150mm tubing (B). Selection of dual-stream mode in LeadScape software allows automatic scheduling of injector and diverter valves (C).

Reconfiguration of an *in vitro* screen

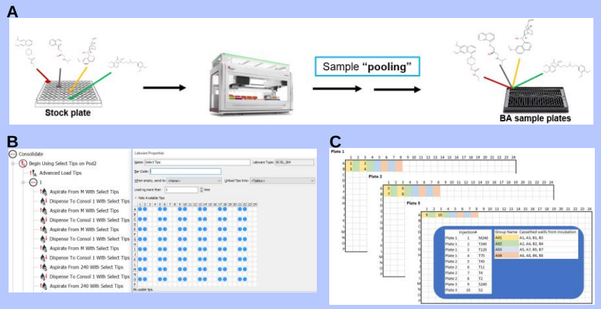


Figure 3a-c: HHEP *in vitro* clearance screen was reconfigured for post-assay sample pooling, resulting in assay samples containing n=4 analytes per each timepoint (A). The sample-pooling operation, scripted and automated on a Biomek i7 liquid handler, utilizes Tip Select feature for execution (B). Whereas the legacy HHEP assay resulted in 11x plates for bioanalysis, sample-pooling enables multiple timepoints to be consolidated per 384-well plate (also automated on Biomek i7). The reconfigured assay now delivers only 3x 384-well plates per run (C).

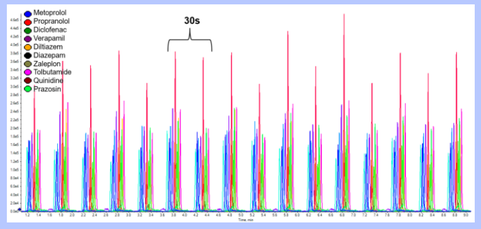
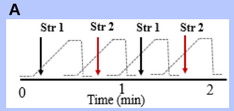


Figure 4: Multi-injected, overlayed XIC of dual-stream µfLC-MS/MS analysis of standard cocktail shows good concordance between injection streams in terms of peak area, retention time, and chromatographic reproducibility. Total injection cycle time is 60s per stream, with each stream is directed to the mass spectrometer every 30s. Agilent pumps were programmed with identical linear gradient time programs: 3-85%B over 31s, and 29s re-equilibration. Mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in acetonitrile.



Group Name	Stream	Compound ID	Compound Lot
A01	Stream 1	Compound 1	Compound 1_batch 1
A01	Stream 1	Compound 2	Compound 2_batch 1
A01	Stream 1	Compound 3	Compound 3_batch 1
A01	Stream 1	Compound 4	Compound 4_batch 1
A02	Stream 2	Compound 5	Compound 5_batch 1
A02	Stream 2	Compound 6	Compound 6_batch 1
A02	Stream 2	Compound 7	Compound 7_batch 1
A02	Stream 2	Compound 8	Compound 8_batch 1

Figure 5a-c: The system was configured for dual-stream analysis leveraging *Duality* feature in LeadScape software, enabling each stream to analyze entirely different sample sets and reduce potential variability to impact clearance determinations (A). Text file containing group name, stream assignments and compound identifier associated analytes with each injection stream, and were imported directly into LeadScape (B). Discrete timepoint samples containing pooled analytes were simultaneously analyzed in dual-stream mode. Data is reviewed using a batch data file that associates analyte identifier with stream selection, enabling deconvolution and review of grouped analytes for each stream (C).

Operational impact of optimized screen

	Endpoints /sample	Throughput (sec/endpoint)	Sample plates/week	FTE	BA runtime	Mobile phase/week
Legacy	1	10	66	12 hrs	96 hrs	13.8L
Cassette, dual-stream µfLC-MS/MS	4	7.5	18	6 hrs	51 hrs	0.275L

Table 1: Impact of incorporating dual-stream microflow for HHEP bioanalysis-comparison of metrics across legacy and revised methods. Bioanalytical runtime is reduced by 47%, recouping additional instrument time that can be redeployed for portfolio impact. In addition, sample plate burden is reduced by 3.6-fold, allowing multiple assay instances to occupy a single instrument plate deck. Overall spend is also reduced; whereas laborious remediation and post-processing was required to increase data return in the legacy HHEP bioanalytical process, increased chromatographic coverage afforded by µfLC-MS/MS virtually eliminates this step. A paradigm shift in LC mobile phase consumption was realized: 50x less solvent is consumed compared to the legacy assay format.

Results

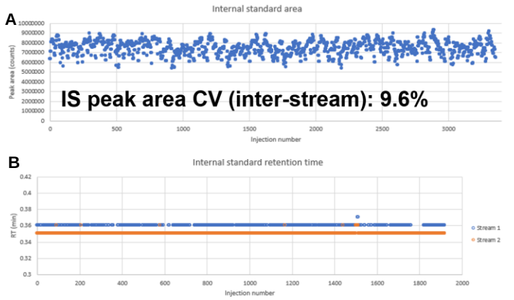


Figure 6a-b: Evaluation of analytical performance from revised HHEP workflow indicate low variability in internal standard response between injection streams over course of 8.5hr run (A). Analysis of internal standard retention times throughout same study indicate excellent system reproducibility and stability at micro-flow rates (B).

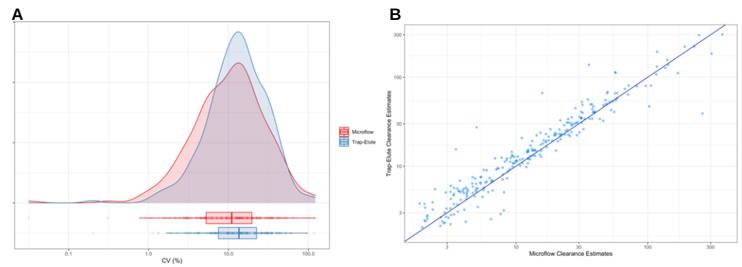


Figure 7a-b: Statistical analysis of apparent intrinsic clearance values (CLint, app) across n=3 back-to-back bioanalytical runs was conducted for both legacy (singleton assay coupled to trap-and-elute analysis) and reconfigured (n=4, post-assay pooling, dual-stream µfLC-MS/MS analysis) assay formats. While a comparable distribution of %CVs was observed, dual-stream µfLC-MS/MS was found to deliver a notable improvement overall (A). Resultant CLint, app estimates were also compared across assay methodologies-a plot of n=341 CLint values from both methods indicates strong concordance, R=0.955 (B).

Conclusions

- ✓ Dual-stream microflow LC-MS/MS was developed to enhance bioanalytical throughput and capacity while maintaining key performance attributes.
- ✓ HHEP *in vitro* clearance assay was reconfigured with fully-automated post-assay sample pooling (n=4 analytes/well) coupled with dual-stream microflow LC-MS/MS analysis.
- ✓ LeadScape software provided fully-integrated setup of bioanalytical methods and optimized data deconvolution.
- ✓ Very good analytical performance and stability were observed, and analysis of resultant CLint, app values revealed a statistically-significant improvement to precision compared to legacy HHEP workflow.

Acknowledgements

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