OPEN ACCESS LC/MS/MS QUANTITATION USING AN ULTRA-HIGH PRESSURE CHROMATOGRAPHY SYSTEM TO BOOST LABORATORY THROUGHPUT AND MAINTAIN DATA QUALITY

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INTRODUCTION

Open access, or walk-up, LC/MS is used routinely in drug discovery for the confirmation of compound synthesis. A similar approach can be applied to the acquisition of ADME data in a high-throughput discovery environment. We present a high throughput LC/MS/MS approach, which uses a novel combination of UPLC together with an open access user interface to increase throughput while maintaining data quality.

In this paper, 10 compounds (shown in Figure 1 below) were run using this automated approach by both UPLC/MS/MS and HPLC/MS/MS to assess the differences between these two techniques.

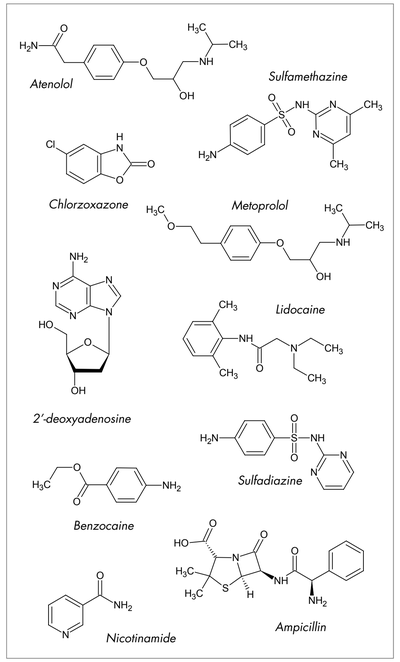

Figure 1. Chemical structures of the ten test compounds.

METHODS

Sample Preparation

Samples were prepared from stock solutions of each compound made up at 1.0mg/mL in 1:1 Water/Methanol. Stock solutions were diluted to 10µg/mL in 95:5 Water/Methanol for use as optimization solutions. Spiking solutions were prepared at the concentrations shown in Table 1. Separate calibration curves for each compound were prepared in pooled sodium heparin human plasma as follows:

- 100µL plasma added to 100µL spiking solution and vortexed
- 300µL acetonitrile added to crash proteins, sample vortexed
- Samples spun down at 13,000rpm for 5 minutes to pellet precipitate
- 100µL of supernatant added to 100µL water and vortexed, this solution is used for injection.

Spike Conc. (ng/mL)	Plasma Equivalent Conc. (ng/mL)	Extract Conc. (ng/mL)	Amount injected on column (5µL inj.)
2.5	1.25	0.25	1.25pg
5	2.5	0.5	2.5pg
25	12.5	2.5	12.5pg
50	25	5	25pg
250	125	25	125pg
500	250	50	250pg
2500	1250	250	1250pg

Table 1. Spiking solution concentrations and equivalents.

HPLC Conditions

Instrumentation: Waters ACQUITY™ UPLC™ System
Column: Waters XBridge™ C_{18}, 2.1 x 50mm, 3.5µm
Eluent A: 0.1% Formic Acid in Water
Eluent B: 0.1% Formic Acid in Methanol
Flow rate: 0.35 mL/min
Gradient:

Time (min)	%A	%B	Curve
0	98	2	-
0.3	98	2	6
3.3	2	98	6
3.6	98	2	11

Run time: 4.5 minutes
Column Temp: 55°C
Injection Volume: 5µL

UPLC Conditions

Instrumentation: Waters ACQUITY™ UPLC™ System
Column: Waters ACQUITY UPLC™ BEH C_{18}, 2.1 x 50mm, 1.7µm
Eluent A: 0.1% Formic Acid in Water
Eluent B: 0.1% Formic Acid in Methanol
Flow rate: 0.7 mL/min
Gradient:

Time (min)	%A	%B	Curve
0	98	2	-
0.1	98	2	6
1.1	2	98	6
1.2	98	2	11

Run time: 1.5 minutes
Column Temp 55°C
Injection Volume: 5µl

MS Conditions

Instrumentation	Waters Quattro Premier XE™
Capillary Voltage	1.5kV
Desolvation Temperature	350°C
Desolvation Gas Flow	1000L/hour
Cone Gas Flow	50L/hour
Collision Gas Pressure	3.5e-3 mbar (argon)

All other conditions generated by QuanOptimise during the optimization stage of the analysis.

EXPERIMENTAL DESIGN

QuanOptimise and OpenLynx application managers were configured to accept a list of compounds and their molecular weights as well a list of samples to be analyzed. These lists are input into the open access interface in either a tab-delimited text file format or copied and pasted into the application from a program such as Microsoft Excel. The QuanOptimise software then uses the optimization samples in the compound list to generate optimum conditions for the analysis of the samples as well as appropriate MRM methods and quantitation methods to run the analysis list.

Optimization was carried out using a 1minute isocratic method (at 98% Eluent B) through the column at 0.3mL/minute, all temperature, eluent and MS conditions are as specified above. The software automatically optimizes the cone voltage (CV), ion mode, (positive or negative ion), product ion selection and collision energy (CE) from this run. MRM methods are then created for each compound (or group of compounds) and a quantitation method is automatically generated for data processing. The optimization results for all compounds used are displayed in Table 2.

For each compound we prepared 2 replicate extractions of each calibration point in human plasma and 2 blank extractions, the analysis list comprised of four blank injections and two 7 point calibration curves for each compound. This list was run in an automated fashion after the optimization run was complete.

Analysis of Atenolol and Metoprolol was repeated by HPLC/MS/MS using the same samples and eluent as prepared for the UPLC/MS/MS run

Compound	*Transition*	*Ion Mode*	*CV*	*CE*
Atenolol	267.1 > 144.9	ESP +ve	25	30
Metoprolol	268.2 > 115.8	ESP +ve	10	25
Sulfamethazine	279.1 > 185.9	ESP +ve	25	20
Chlorzoxazone	167.7 > 131.8	ESP -ve	35	25
2-deoxyadenosine	252.0 > 135.8	ESP +ve	20	15
Lidocaine	235.1 > 85.8	ESP +ve	20	20
Sulfadiazine	250.9 > 155.8	ESP +ve	25	15
Ampicillin	350.1 > 105.8	ESP +ve	25	20
Benzocaine	165.8 > 137.8	ESP +ve	25	15
Nicotinamide	122.7 > 79.8	ESP +ve	35	20

Table 2. Optimization Results for all compounds.

RESULTS

All data produced was processed in an automated fashion by the software after completion of the analysis list.

All 10 compounds produced good linearity with r^2 values of greater than 0.98 with all but three giving better than 0.995 by UPLC/MS/MS. Linear range of each compound was at least 0.25—50 ng/mL by UPLC/MS/MS. Full details of these figures are shown in Table 2 . Calibration curves for each compound by UPLC/MS/MS are shown in Figure 2 below.

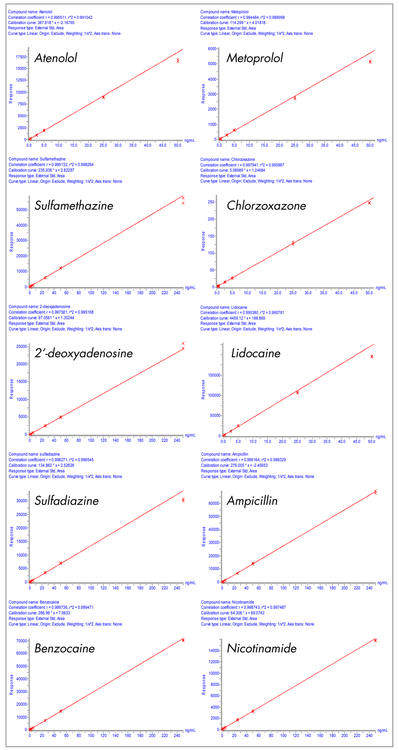

Figure 2. Calibration curves for tested compounds by UPLC/MS/MS.

Compound	*Linear Dynamic Range (extract concentration, ng/mL)*	*r^2*
Atenolol	0.25—50	0.9910
Metoprolol	0.25—50	0.9890
Sulfamethazine	0.25—250	0.9983
Chlorzoxazone	0.25—50	0.9959
2-deoxyadenosine	0.25—250	0.9952
Lidocaine	0.25—50	0.9908
Sulfadiazine	0.25—250	0.9965
Ampicillin	0.25—250	0.9983
Benzocaine	0.25—250	0.9995
Nicotinamide	0.25—250	0.9975

Table 2. Linear Dynamic Range and r^2 for all compounds by UPLC/MS/MS.

Compound	*Linear Dynamic Range (extract concentration, ng/mL)*	*r^2*
Atenolol	0.25—25	0.9932
Metoprolol	0.25—50	0.9810

Table 3. Linear Dynamic Range and r^2 for Atenolol and Metoprolol by HPLC/MS/MS.

The HPLC/MS/MS runs for Atenolol and Metoprolol gave comparable linearity and to the UPLC/MS/MS results although Atenolol did exhibit slightly lower linear range by HPLC/MS/MS. Linear range and r^2 values for these two runs are shown in table 3 above.

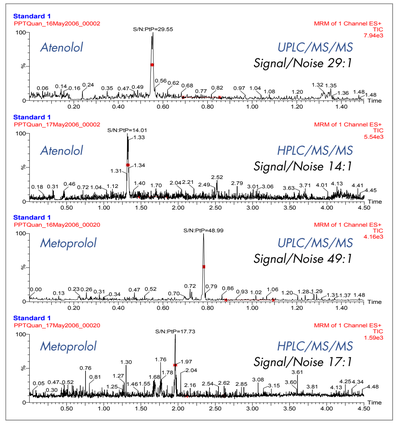

Figure 3. Signal/Noise comparison for the 0.25ng/mL standard of Atenolol and Metoprolol by UPLC/MS/MS and HPLC/MS/MS.

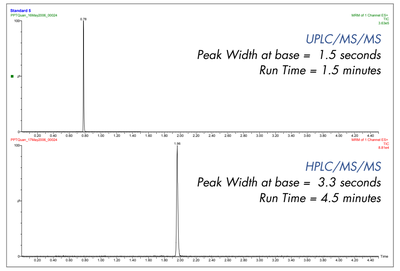

Figure 4. Speed and peak width comparison for the 25ng/mL standard of Metoprolol by UPLC/MS/MS and HPLC/MS/MS.

DISCUSSION

The methodology used to generate the data shown in this paper is designed to allow new and inexperienced users of triple quadrupole instrumentation to generate good quality experimental data with minimal user input. It allows experienced users a simple interface to speed up the submission of routine sample sets.

We have used a generic 1.5 minute UPLC gradient along with automation software to allow the user to simply input a list of compound information and a list of samples, the software will then automatically optimize the response for each compound, generate the necessary methods, run a sample list and automatically generate a quantitation results file.

We have shown good linearity and dynamic range (r^2 > 0.989 and dynamic range > 0.25—50ng/mL) for protein precipitated human plasma samples tested by UPLC/MS/MS.

CONCLUSION

- **We have demonstrated the feasibility of generating good quality quantitative data using a highly automated UPLC/MS/MS methodology from samples in dirty matrix.**
- **From the data available UPLC/MS/MS gave between 2x and 3x improvement in signal/noise when compared to HPLC/MS/MS.**
- **The UPLC/MS/MS gave 3x the throughput of the HPLC/MS/MS method while giving highly comparable results.**

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