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EVALUATION OF UPLC/MS/MS FOR THE VALIDATED BIOANALYTICAL QUANTIFICATION OF AMITRIPTYLINE IN HUMAN PLASMA

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INTRODUCTION

In this work we present a method of analysis for Amitriptyline, a common anti-depressant, in human plasma that has been validated on a HPLC/MS/MS system to the strict guidelines required by the FDA. This analysis was then compared to a method developed for a new UPLC™/MS/MS system that has made the analysis more sensitive and faster. Incorporating ultra performance liquid chromatography technology with the latest technology in tandem quadrupole mass spectrometry has resulted in a faster and more sensitive approach to quantitative bioanalysis.

AIMS

The goal of these experiments was to develop a quantitative method for Amitriptyline in human plasma using Ultra Performance Liquid Chromatography (UPLC™) coupled to a tandem quadrupole mass spectrometer. UPLC is a novel technique that utilises columns packed with 1.7 µm particles, operated at high linear velocities resulting in increased chromatographic resolution and peak height compared to conventional HPLC. This technique has never previously been applied to a validated quantitative bioanalysis assay.

EXPERIMENTAL

SAMPLE EXTRACTION PROCEDURE

1. The method consisted of mixing human plasma (varying amounts depending on the standard used see Table 1) with internal standard (Doxepin at 100 pg/µL, 10 µL).
2. The resulting mixture was vortex mixed (*ca* 10 seconds) and centrifuged (*ca* 3000 rpm, *ca* 3 minutes).
3. The supernatant was placed into a glass vial and injected onto a Waters® Symmetry® C_{18} (100 x 2.1 mm) HPLC column.
4. A 10 µL injection of the extract was run by gradient reversed phase chromatography with a mobile phase comprised of water (0.1% formic acid): acetonitrile (0.1% formic acid) at a flow rate of 0.3 mL/minute.

PREPARATION OF STANDARD SOLUTIONS

Amitriptyline primary stock solution (1mg/mL) and working standard solutions

A primary stock solution of Amitriptyline was prepared at a concentration of 1 mg/mL by accurately weighing out 5 mg of Amitriptyline into a flask and dissolving in 5 mL of mobile phase.

Internal standard primary stock solution (100 pg/µL)

Internal standard solution was prepared by diluting a stock solution of Doxepin (1 mg/mL) 1 in 10 with acetonitrile/water (0.1% formic acid), 50/50, v/v.

Amitriptyline secondary stock solutions (100 ng/µL, 1 ng/µL and 100 pg/µL)

The 1 mg/mL solution was diluted 1 in 10 with 50/50 v/v, acetonitrile/water to produce 100 ng/µL. Further dilutions were made to produce the 1 ng/µL, 100 pg/µL and 10 pg/µL.

PREPARATION OF HUMAN PLASMA CALIBRATION STANDARDS

Aliquots of Amitriptyline working standard solutions were added to blank human plasma on the day of the analysis to produce a calibration line at the concentrations stipulated below.

Concentration of Amitriptyline standard solution (ng/µL)	Volume of standard solution (µL)	Volume of Doxepin internal standard (µL)	Volume of plasma (µL) + 500 µl of acetonitrile	Final concentration (pg/µL)
100	50	10	440	5000*
100	10	10	480	1000*
100	5	10	485	500*
1	100	10	390	100
1	50	10	440	50
1	10	10	480	10
0.1	50	10	440	5
0.1	10	10	480	1
0.01	50	10	440	0.5**
0.01	10	10	480	0.1
0.001	50	10	440	0.05**
0.001	10	10	480	0.01**

Table 1. Detail for the preparation of human plasma calibration standards.
** Used only for the LC/MS/MS calibration line.*
*** Used only for the UPLC/MS/MS calibration line.*

PREPARATION OF QUALITY CONTROL SAMPLES

The QC samples were divided into suitable volumes and analysed on preparation. The remaining was stored at –22 °C until used for analysis.

Standard concentration used (ng/µL)	Volume of standard solution used (µL)	Volume of plasma (µL) + 500 µl of acetonitrile	Volume of Doxepin internal standard (µL)	Final concentration (pg/µL)
0.01	30	460	10	0.3**
0.1	30	460	10	3**
1	30	460	10	30*
10	30	460	10	300*
100	30	460	10	3000*

Table 2. Detail for the preparation of quality control samples.
** Used only for the LC/MS/MS calibration line.*
*** Used only for the UPLC/MS/MS calibration line.*

LC/MS/MS

LC system: Waters® 1525µ Binary HPLC Pump with a 2777 Sample Manager

Column: Waters Symmetry® C_{18}, 3.5 µm, 2.1 x 100 mm

Isocratic: Mobile phase A: Water (30%)
Mobile phase B: Acetonitrile (70%)

Injection volume: 10 µL

UPLC/MS/MS

LC system: Waters ACQUITY UPLC™

Column: Waters ACQUITY BEH C_{18}, 1.7 µm, 2.1 x 100 mm

Gradient: Mobile phase A: Water
Mobile phase B: Acetonitrile

Time (min)	Flow Rate (mL /min)	% A	% B
Initial	0.3	95	5
0.50	0.3	5	95
1.50	0.3	5	95
1.60	0.3	95	0 5

Injection volume: 5 µL

MASS SPECTROMETRY CONDITIONS

MS System: Waters Micromass® Quattro Premier™

MRM Transition	Dwell (secs)	Cone Volt.	Col.Energy
278.30 > 91.00	0.05	25.0	26.0
280.50 > 106.90	0.05	22.0	23.0

RESULTS

(A). LINEARITY

All calibration lines run over a five-day calibration gave correlation coefficients of greater than r^2 0.99 with all standards within +/-15% deviation. Examples of two such calibration lines can be seen in Figure 1. This figure shows that the UPLC/MS/MS calibration line is equivalent to the standard HPLC/MS/MS calibration line in terms of the correlation coefficients. The UPLC/MS/MS method differs in the linearity as it covers the range of 0.01 to 100 pg/µL where as the standard HPC/MS/MS is over the range of 0.5 to 500 pg/µL. This is due to the extra sensitivity that is obtained by running UPLC with the Quattro Premier. The top standard was too concentrated and saturated the detector. Therefore, the top standard was reduced to 100 pg/µL with the whole linear range going down to 0.01 pg/µL and maintaining the four orders of linear magnitude. Clearly, with the UPLC-Quattro Premier we have shown that increased sensitivity can be achieved while maintaining linearity over 4 orders of magnitude.

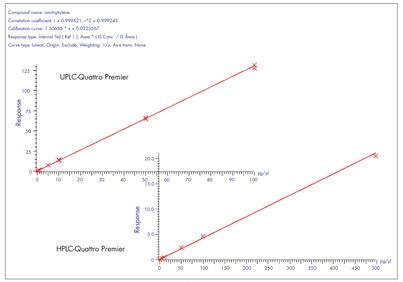

Figure 1. A comparison of the linearity of the UPLC-Quattro Premier with the standard HPLC-Quattro Premier.

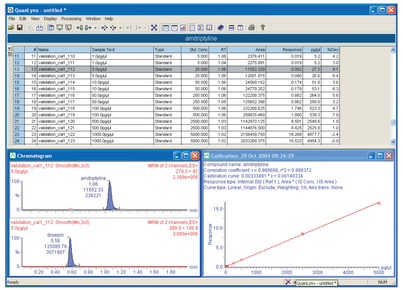

Figure 2. Quantification results window.

(B) QUANTIFICATION

The system approach to quantitative bioanalysis incorporates quantification software that fully supports UPLC and the Quattro Premier. The results shown in Figure 2 give excellent back calculated concentrations with deviations from the theoretical concentrations being +/-10%. This shows good correlation to the strict criteria as stipulated in GLP environments.

(C). SENSITIVITY AND CHROMATOGRAPHIC RESOLUTION

The sensitivity differences have been highlighted on the QC 30pg/µL samples. In Figure 3 the signal to noise ratio is used as a guide to the relative differences in sensitivity that can be achieved. With the standard HPLC-Quattro Premier a relatively good signal to noise ratio of approximately 900:1 is achieved. This was compared to the UPLC-Quattro Premier and the result was 4500:1—an increase of five times in sensitivity with an increase of ten times in overall signal intensity.

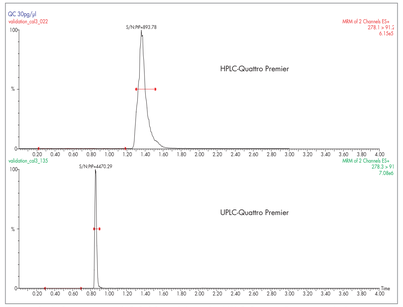

Figure 3. A Comparison of QC 30 pg/µL on the UPLC-Quattro Premier with the HPLC-Quattro Premier.

The figure above shows improvements in speed, sensitivity and resolution for the analytical method when using the ACQUITY UPLC System.

The main gains in sensitivity and speed come from the UPLC-Quattro Premier System's ability to use 1.7 µm particle stationary phases at elevated linear velocities. The result is a peak width of 3 seconds at base compared with the standard chromatographic peak width for Amitriptyline of 15 seconds at base. From the focussing of the peak on the column, the tandem mass spectrometer now has a new problem. With standard peak widths, the mass spectrometer needs to sample across the peak and achieve a minimum of 15 sampling points in order to achieve a Gaussian peak that is representative. At very narrow peak widths this is made more difficult, as the narrower the peak the faster the mass spectrometer needs to sample. With the Quattro Premier this problem is overcome by incorporating the T-Wave™ device that allows 30 sampling points to be collected within the 3-second peak width.

(D). ROBUSTNESS

For all good validated studies a robust method is required. The UPLC-Quattro Premier method was evaluated by running 200 QC's over a 24-hour period. This experiment was designed to see how robust the whole system and methodology was with complex matrices. The overall percentage variation expressed as a coefficient of variation (CV), was 2.9% as can be seen in Figure 4.

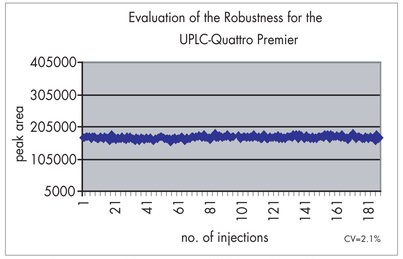

Figure 4. Evaluation of the Robustness for the UPLC-Quattro Premier Validated Method.

CONCLUSIONS

The calibration lines all gave coefficients of determination of better than 0.995 over a linear range of 0.1 pg/µL to 5000 pg/µL. Back-calculated values for calibration and QC standards were all within 15%, including at the LOQ, thereby meeting the accepted criteria for bioanalytical method validation. No deterioration of column performance was observed for the duration of the analysis. Comparison with the HPLC/MS/MS data showed that, for the same analysis time, the chromatographic resolution was improved.

1.7 µm particle packed columns operated at elevated pressures have been used for the quantitative analysis of Amitriptyline in protein precipitated human plasma. Improved chromatographic resolution and sensitivity were observed compared to an equivalent HPLC/MS/MS method.