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AUTOMATED ON-LINE SOLID PHASE EXTRACTION CHROMATOGRAPHY TECHNIQUE FOR THE DIRECT ANALYSIS OF TRICYCLIC ANTIDEPRESSANTS IN BIOLOGICAL MATRICES

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INTRODUCTION

NCE's (new chemical entities) are becoming more potent, in turn the administered dosages are decreasing, which leads to lower limits of detection required (sub ng/mL). Biological samples from *in vivo* studies need to be analysed rapidly throughout a NCE lifecycle. Consequently the analysis of biological samples must be very rapid yet remain selective and sensitive for the compounds of interest. Due to these pressures LC/MS/MS has become the standard analytical tool for the determination of NCE's in complex matrices. The analysis of NCE's and their metabolites in biological matrices requires sufficient sample preparation prior to separation, detection and quantification to reach the low detection limits by overcoming ion suppression effects, for example. Sample pre-treatment can be time consuming, repetitive and prone to human error due to the large number of samples required for analysis. This means that a generic approach with the direct injection of biological matrices using an on-line system is desirable[1]. In this paper we discuss the use of a new automated software platform for on-line sample extraction and analysis in a totally integrated manner.

The aim of this study was to evaluate an on-line sample extraction system for the direct analysis of tricyclic antidepressants (TCAs) in biological matrices. Selectivity, linearity, precision, recovery and limit of quantification were studied

EXPERIMENTAL

Methodology

The tandem mass spectrometry system was configured using multiple pumps and switching valves in order to perform on-line sample extraction of biological samples under a single software platform as outlined in Figures 1–4.

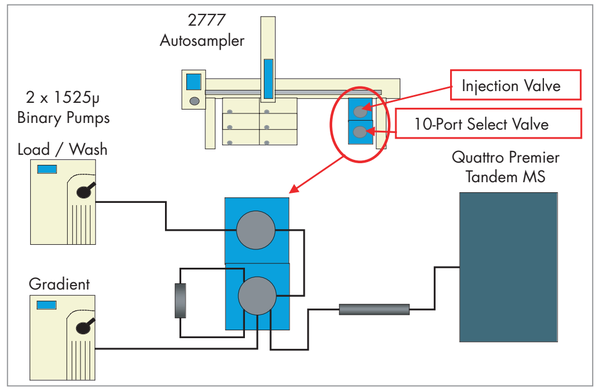

Figure 1. Schematic of the hardware configuration.

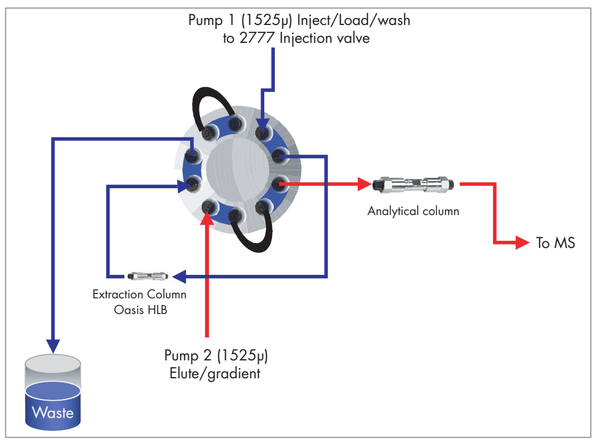

Figure 2. Configuration of the 10-port switching valve.

Hardware Set Up

Figure 1 shows a schematic of the on-line sample preparation configuration. Figure 2 shows the configuration of the 10-port switching valve. The sample extraction procedure and analysis is outlined below:

In the OFF position (dark blue pathway) the diluted plasma is loaded onto the Oasis® HLB in aqueous mobile phase at a high flow rate (2 mL/min) with the flow directed towards waste.

The extraction column is washed in 5% organic at 2 mL/min.

Plasma matrix is directed towards waste and the analytes are retained on the sorbent by reverse-phase mechanisms.

The valve is switched to the ON position so that the extraction column and analytical column are in line.

Start the organic gradient (5–95% organic) at typical LC/MS flow rates (0.3 mL/min).

Analytes are released from the sorbent onto analytical column where they are focused/ separated before detection by MS/MS.

Re-equilibration.

Software Set Up

Multi pump functionality was enabled in MassLynx. Using this software it is possible to control two gradient pumps and up to three isocratic pumps.

Two Waters 1525µ pumps and a Waters 2777 autosampler were used in following studies along with a two position 10-port valve, which was controlled via contact closure through one of the pumps. An Oasis® HLB 2.1 x 20mm column was used for extraction and a Symmetry® C_{18} 3.5 µm 2.1 x 100 mm column for sample separation.

The two Waters 1525µ pumps were set up as follows:

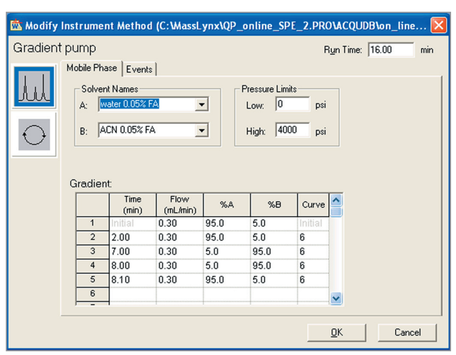

Figure 3. Pump 1 (Gradient pump): Analytical column separation.

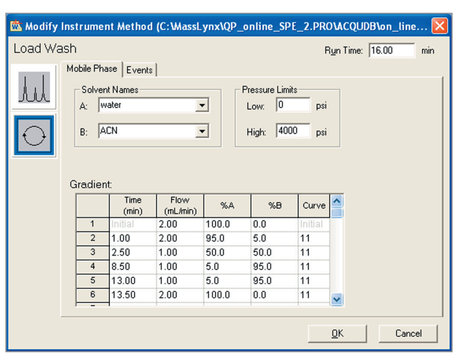

Figure 4. Pump 2 (Load Wash): Load in 100% water for 1 min, wash for 1 min in 5% acetonitrile.

Sample Preparation

Stock solutions (1 mg/mL) of verapamil, doxepin and amitriptyline were prepared in methanol.

- *Calibration curve in solution*
  Subsequent dilutions were made in mobile phase to produce a serial dilution of doxepin and amitriptyline (1 µg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng, 1 ng, 500 pg/mL)
- *Calibration curve in protein-precipitated plasma*
  Drug free human plasma was protein precipitated by adding acetonitrile (2 organic: 1 plasma). The mixture was centrifuged at 13,000 rpm for 15 minutes. Subsequent dilutions were made in supernatant to produce a serial dilution of doxepin and amitriptyline (1 µg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng, 1 ng, 500 pg/mL).
- *Calibration curve in diluted plasma*
  Drug free human plasma was diluted to reduce the viscosity by adding HPLC grade water (2 water: 1 plasma). Subsequent dilutions were made in the diluted plasma to produce a serial dilution of doxepin and amitriptyline (1 µg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng, 1 ng, 500 pg/mL).

Verapamil was used as the internal standard spiked at the 10 ng/mL level and quality control (QC) samples (n=20) were prepared at the 100 ng/mL level for all calibration curves. The QC samples were treated in the same way as the standards. Each standard was analysed in triplicate.

MS Conditions

Instrument:	Waters® Micromass® Quattro Premier™
Ionisation:	Electrospray positive ion
Capillary:	3.20 kV
Source Temperature:	120 °C
Desolvation Temperature:	350 °C
Argon Gas Pressure:	3.33 x 10^{-3} mbar
Inter Scan delay:	0.01 secs
Inter Channel delay:	0.01 secs

Multiple Reaction Monitoring

Compound	MRM transitions	Dwell	Cone Volt	Col. Energy
Amitriptyline	278.10 > 91.20	0.05	30.0	27.0
Doxepin	280.10 > 107.10	0.05	25.0	22.0
Verapamil (IS)	455.20 > 165.10	0.05	40.0	30.0

HPLC Conditions

Injection volume:	5 µL
Extraction column:	Oasis HLB, 25 µ m, 2.1 x 20 mm
Analytical column:	Symmetry C_{18} 3.5 µm 2.1 x 100 mm

Pump 1 (Gradient)		*Pump 2 (Load Wash)*	
Solvents:	A: Water 0.05% formic acid	Solvents:	A: Water
	B: Acetonitrile 0.05% formic acid		B: Acetonitrile
Gradient:	As shown in Figure 3	Gradient:	As shown in Figure 4

RESULTS

The selectivity of the method was determined by comparing the blank plasma to the plasma spiked with the mixture of analytes and the internal standard. No interfering compounds were detected at the same retention times as the studied compounds.

The data were processed using QuanLynx™ quantification software, using the ApexTrack™ integration algorithm. A calibration curve of amitriptyline and doxepin in diluted plasma over the concentration range 0.5–1000 ng/mL were generated. A linear curve fit was applied with a 1/x weighting. The correlation coefficient (R^2) was > 0.998 for both compounds and the calculated concentration % deviations are all within FDA guidelines for bioanalytical method validation. Figure 5 shows the QuanLynx report for the calibration curve of amitriptyline in diluted plasma over the concentration range 0.5–1000 ng/mL.

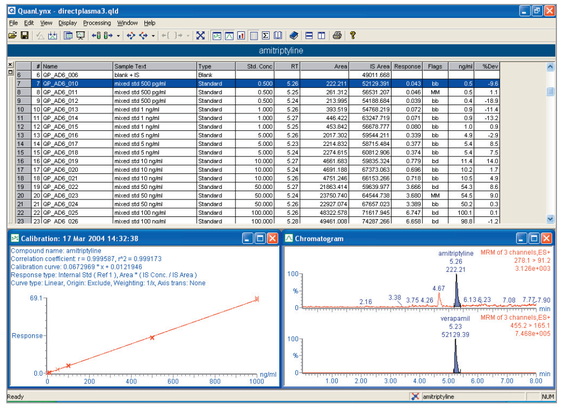

Figure 5. QuanLynx™ report of Amitriptyline in diluted plasma.

The LOQ for amitriptyline in diluted plasma was 2.5 pg on column (500 pg/mL injecting 5 µL) where the signal to noise (peak to peak) was 13:1 (calculated by ignoring scans outside 1 standard deviation).

The 20 QC samples analysed at the 100 ng/mL level yielded a relative standard deviation of 4.31% for amitriptyline.

The extraction efficiencies of doxepin and amitriptyline are shown in Figure 6 for the protein-precipitated plasma and diluted plasma studies.

The carryover of the assay was determined as the blank sample prior to the LOQ was <13% and <1% following the top standard.

Compound	Extraction efficiencies in protein-precipitated plasma	Extraction efficiencies in diluted plasma
Amitriptyline	70%	32%
Doxepin	99%	38%

Figure 6. Extraction efficiencies.

DISCUSSION

The results demonstrate that this automated on-line solid phase extraction tandem mass spectrometry system is suitable for the direct analysis of biological matrices.

The system is easy to set up and operate under a single software platform.

This generic SPE procedure decreases the need for off-line sample handling.

The generic method developed showed good selectivity for the analytes and internal standard.

The generic extraction and separation methods were used to obtain LOQs for both TCAs at the 2.5 pg on column by injecting 5 µL of a 500 pg/mL sample.

The calibration curves for doxepin and amitriptyline were linear over the range 0.5–1000 ng/mL with the correlation coefficients >0.998 and the calculated % deviations all within GLP guidelines.

The precision of the method was demonstrated by the QC samples having a % RSD of <5%.

CONCLUSION

We have demonstrated that by using a generic on-line extraction method coupled with the Quattro Premier™ tandem mass spectrometer, LOQs of <1 ng/mL can be reached when directly analysing TCAs in diluted plasma.

REFERENCES

1. G. Hopfgartner and E. Bourgogne 'Quantitative high-throughput analysis of drugs in biological matrices by mass spectrometry' Mass Spectrometry Reviews, 2003, 22, 195–214.