High Throughput LC/MS/MS Analysis of Serotonin Re-uptake Inhibitors from Protein Precipitated Plasma Samples

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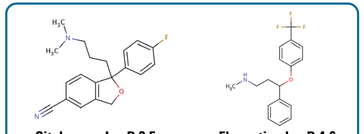
Introduction

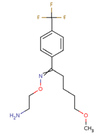
Clinical researchers interested in the quantitation of selective serotonin reuptake inhibitors (SSRIs) would benefit from the development of an analytical method with short analysis times, high sensitivity and excellent reliability. The removal of protein from plasma samples is a critical step to achieving these goals. Standard protein precipitation followed by centrifugation is not only slow, but does not adequately remove protein, resulting in unacceptably high variation in quantitation. The use of a protein precipitation plate facilitates quick, easy, and near complete removal of proteins which interfere with the analysis of SSRIs in plasma. This improved protein removal lowers variation to an acceptable level, providing confidence in quantitation. The plate's non-drip design also allows for the automation of protein precipitation thereby drastically increasing throughput while improving sensitivity and reproducibility.

Figure 1. Analytes

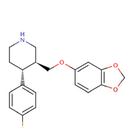


Citalopram LogP 3.5

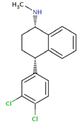
Fluoxetine LogP 4.6



Fluvoxamine LogP 3.2



Paroxetine LogP 3.6



Sertraline LogP 5.1

Standards were ordered from Sigma-Aldrich and made into 1.0 mg/mL stock solutions in methanol from which the appropriate spiking solutions were produced.

Experimental

Human plasma was spiked to a concentration of 5.0 ng/ml & 500 ng/ml of Citalopram, Fluoxetine, Fluvoxamine, Paroxetine, and Sertraline. Fluoxetine-D6 was used as an internal standard.

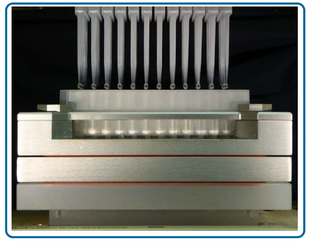
Standard Protein Precipitation

400µl of acetonitrile was added to a centrifuge tube, followed by 100µl of spiked Human plasma. The centrifuge tube was vortexed and inverted followed by centrifugation for 6 min at 15000rpm.

Automated Protein Precipitation

A Tomtec Quadra 96 liquid handling robot was used with a new non-drip filtration plate (Figure 2) to perform an automated protein precipitation. The robot was programmed to add 400µl of acetonitrile to the plate followed by 100µl of spiked Human plasma. The entire sample volume was mixed 5 times, in well, by the robot. A vacuum of 15 in Hg was then applied.

Figure 2. Automated Analysis Setup



Preparation for Analysis

The eluent from both procedures was diluted 1:1 with mobile phase then spiked with internal standard to a concentration of 10µg/ml Fluoxetine-D6. Samples were analyzed for protein removal, analyte peak area and precision of quantitation.

Analysis of Protein Removal

The Bradford protein assay (Fisher) was used as a quick and easy check of protein removal from both samples. The eluent of 24 wells was pooled and concentrated. Eight samples were precipitated using centrifugation, pooled and concentrated. Both pools were measured in triplicate.

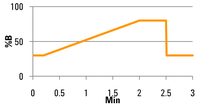
Experimental

LC Conditions

Column – Poroshell C18 2.7 µm 50 mm x 3.0 mm
Instrument: Agilent 1290/6460 QQQ

Mobile Phase
A: 0.1% Aqueous Formic Acid
B: ACN

Mobile Phase Gradient



Pump Program
Flow rate 400 µL/ min.
t_0 A: 70%, B: 30%
$t_{0.2-2.0}$ A: 20%, B: 80%
$t_{2.51-3.00}$ A: 70%, B: 30%
Run Time = 3:00 minutes.

MS Conditions

Source: AJS ESI+
Gas Temp: 350 °C
Gas Flow: 12 l/min
Nebulizer: 25 psi

Table 1. MRM Transitions

Compound	Precursor Ion	Product Ion	CE (V)
Citalopram	325.2	109.1	24
Fluoxetine	310.1	148.1	0
Fluoxetine-D6	316.2	154.2	0
Fluvoxamine	319.2	258.1	4
Paroxetine	330.2	192.1	16
Sertraline	306.1	159.0	24

Results and Discussion

Protein concentrations for pooled samples were calculated using a standard calibration curve (R^2 = 0.998). Average percent error of the calibration curve was 1.7%. Use of the non-drip protein precipitation plate showed considerably improved protein removal compared to that of standard protein precipitation (Figure 3).

SSRIs were quantified using extracted calibration curves ranging from 0.5 ng/mL to 1000 ng/mL. All analytes demonstrated a first order regression with a correlation coefficient of 0.999 (Figure 4).

Plasma was spiked with 5 SSRIs at 5.0 ng/mL and 500 ng/mL. Eight replicates of each spiking level were processed via standard protein precipitation and the non-drip plate. Results were calculated based on a first order regression of a standard protein precipitated calibration curve.

RSDs for all analytes were improved using the non-drip plate. When extracted using the non-drip plate, most compounds also showed equivalent or better peak areas when compared to standard protein precipitation samples (Table 2).

Figure 3. Protein Content per Well & Sample

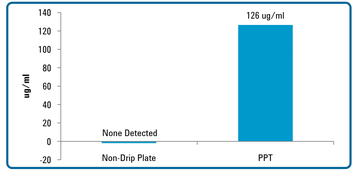
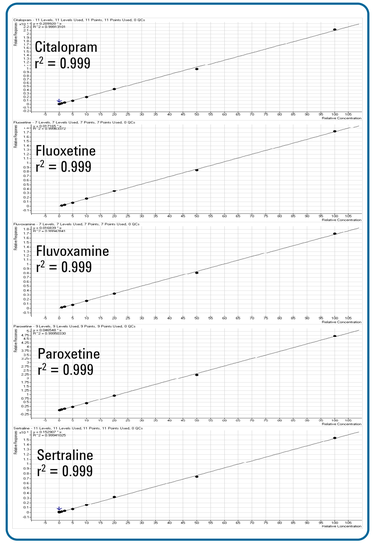


Figure 4. Calibration Curves



Results and Discussion

Table 2. Peak Area & Reproducibility

Citalopram (n=8)			
Conc (ng/ml)	*Area (Plate vs PPT)*	*Plate RSD*	*PPT RSD*
5.0	**137%**	**8%**	14%
500.0	**123%**	**8%**	18%

Fluoxetine (n=8)			
Conc (ng/ml)	*Area (Plate vs PPT)*	*Plate RSD*	*PPT RSD*
5.0	103%	12%	13%
500.0	88%	**6%**	13%

Fluvoxamine (n=8)			
Conc (ng/ml)	*Area (Plate vs PPT)*	*Plate RSD*	*PPT RSD*
5.0	**134%**	17%	20%
500.0	**144%**	8%	11%

Paroxetine (n=8)			
Conc (ng/ml)	*Area (Plate vs PPT)*	*Plate RSD*	*PPT RSD*
5.0	99%	15%	16%
500.0	**106%**	11%	12%

Sertraline (n=8)			
Conc (ng/ml)	*Area (Plate vs PPT)*	*Plate RSD*	*PPT RSD*
5.0	82%	11%	13%
500.0	82%	**6%**	14%

Average RSD	
Method	**Average RSD**
Standard Protein Precipitation (n=8)	14%
Non-Drip Plate (n=8)	10%

Figure 5. Time Savings Using Plate

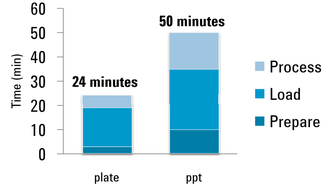
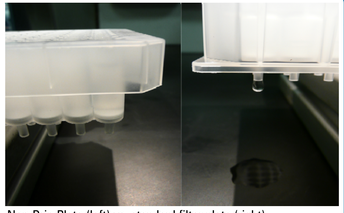


Figure 6. Solvent Retention after 5 mins



Non-Drip Plate (left) vs. standard filter plate (right)

The plate used in this study contains a non-drip design capable of retaining organic solvents. Methanol and acetonitrile were retained for a minimum of 5 mins without any signs of leaking (Figure 6). The non-drip design allows for quick, easy analyses on a large number of samples and is even suitable for automation. Using a non-drip plate, sample preparation times can be cut in half when compared to standard protein precipitation. In this study, preparation of materials dropped from 10 minutes to 3 minutes, loading of samples and reagents fell 9 minutes and the time to process samples sample was reduced from 15 minutes to 5 minutes. A procedure that takes 50 minutes using standard protein precipitation can now be completed in 24 minutes using a non-drip plate (Figure 5).

Conclusion

•Filtration using a non-drip filtration plate provides near complete protein removal compared to that of centrifugation. Protein levels in the post plate eluent were low enough to be undetected.

•The assay was improved using a non-drip filtration plate. RSDs were reduced by 4% over the protein precipitation procedure. Response as measured by peak area was improved for most compounds.

•High throughput analysis of human samples is slow and difficult with standard centrifugation. The use of filtration plates facilitates the high throughput automation of protein precipitation required in the analysis of SSRIs from human samples, and is suitable for a number of analyses.

•A new non-drip filtration plate design allows for the automation of protein precipitation without the mess associated with standard filtration plates.