Altasciences, 575 Armand-Frappier, Laval, Québec, H7V 4B3, Canada
Altasciences.com | contact@altasciences.com

Quantitation of Fenfluramine and Norfenfluramine in Mouse Cerebellum using a Novel SLE Prototype

Vinicio Vasquez[1], Jeff Plomley[1], Milton Furtado[1], Christophe Deckers[2] and Anahita Keyhani[1]

[1]Altasciences, Laval, Quebec, Canada

[2]Agilent Technologies, Montreal, Quebec, Canada

OVERVIEW

NOVEL ASPECT

The first report for the determination of fenfluramine (FNN) and norfenfluramine (NFNN) in brain tissue using a novel SLE prototype.

METHOD

Mouse (CD-1) brain or cerebellum homogenate samples were extracted with an optimized procedure using an Agilent Technologies SLE prototype in the 96-well plate format (160 mg capacity).

RESULTS

The Agilent SLE prototype furnished recoveries of 80% and 72% for FNN and NFNN, respectively. In contrast, recoveries from diatomaceous SLE were 68% (FNN) and 65% (NFNN). Comparison of phospholipid profiles in homogenate extracts from SLE prototype and diatomaceous substrates revealed greater trapping efficiency of these potential suppressors when using the SLE prototype in combination with more highly polar elution solvents.

Intra- and inter-day precision and accuracy batches using the SLE prototype met all acceptance criteria without matrix effect or interference as evaluated from six different donors. Curves were linear with an ULQ of 500.00 pg/g and LOQ of 5.00 pg/g, the latter achieved with S/N ratio of 10:1.

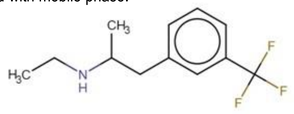
INTRODUCTION

Fenfluramine (FNN) is an anti-epileptic drug whose mechanism of action is poorly understood. In order to study the distribution of FNN and the accumulation of its major metabolite norfenfluramine (NFNN) in mouse cerebellum, it was necessary to develop a sensitive assay given the limitation in tissue mass (ca. 60 mg). Due to the phospholipid-rich nature of brain, it was necessary to deplete as many of these potential ion suppressors as possible in order to minimize accumulation within the LC-MS system. To this end, a novel SLE prototype was evaluated in terms of the efficacy of phospholipid removal, recovery of FNN and NFNN, and assay specificity and matrix effect, all benchmarked against traditional diatomaceous earth sorbent.

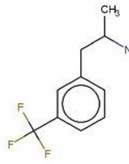
METHODS

SAMPLE PREPARATION

Mouse (CD-1) brain or cerebellum homogenate (10 µL) were fortified with deuterated internal standard (25 µL) and mixed with 5% NH_4OH (125 µL). A 125 µL aliquot was loaded onto the 96w Agilent Technologies SLE prototype (160 mg) and allowed to soak for five minutes prior to elution with 400 µL (x2) of ethyl acetate:dichloromethane (1:1). Elution was finalized by applying positive pressure and extracts were evaporated (50ºC) and reconstituted with mobile phase.



Fenfluramine (FNN)
Chemical Formula: $C_{12}H_{16}F_3N$
Exact Mass: 231.12
Molecular Weight: 231.26
logP: 3.47



Norfenfluramine (NFNN)
Chemical Formula: $C_{10}H_{12}F_3N$
Exact Mass: 203.09
Molecular Weight: 203.20
logP: 2.68

Figure 1. Structures of fenfluramine and norfenfluramine.

CHROMATOGRAPHY

- Shimadzu Nexera X2 UHPLC
- ACE Excel 2 Super C_{18} column
- Mobile phases: 10 mM ammonium bicarbonate pH 10.0 and methanol

MASS SPECTROMETRY

- SCIEX 6500+ Triple Quad , (+) ESI-MRM
  - Fenfluramine: *m/z* 232 > 159
  - Norfenfluramine: *m/z* 204 > 109

RESULTS

RECOVERY

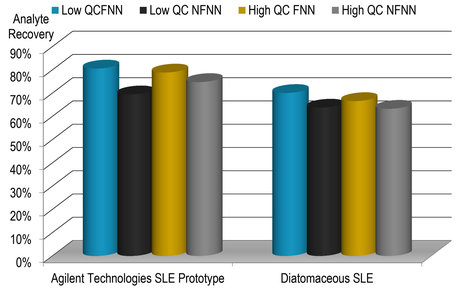


Figure 2. Fenfluramine (FNN) and norfenfluramine (NFNN) recoveries from whole brain homogenate for Low (15.0 pg/g) and High (400.0 pg/g) QC's comparing Agilent SLE prototype against diatomaceous earth.

BETWEEN-RUN PRECISION AND ACCURACY

3 batches, 6 replicates/QC level; SLE prototype; Whole Brain Homogenate

Batch	QC –LOQ 5.00 pg/g		QC-1 15.00 pg/g		QC-2 250.00 pg/g		QC-3 400.00 pg/g	
	FNN	NFNN	FNN	NFNN	FNN	NFNN	FNN	NFNN
Mean	4.80	4.79	14.47	14.76	238.10	243.37	421.27	419.05
S.D.	0.36	0.34	0.97	0.86	14.25	16.68	17.70	17.87
n	18	18	18	18	18	18	18	18
CV [%]	7.5	7.1	6.7	5.8	6.0	6.9	4.2	4.3
Nominal [%]	96.1	95.9	96.5	98.4	95.2	97.3	105.3	104.8

BETWEEN-RUN PRECISION AND ACCURACY

3 batches, 6 replicates/QC level; SLE prototype; Cerebellum Homogenate

Batch	QC-1 15.00 pg/g		QC-3 400.00 pg/g	
	FNN	NFNN	FNN	NFNN
Mean	14.57	14.60	420.77	419.77
S.D.	0.73	0.83	19.47	16.00
n	18	18	18	18
CV [%]	5.0	5.7	4.6	3.8
Nominal [%]	97.1	97.3	105.2	104.9

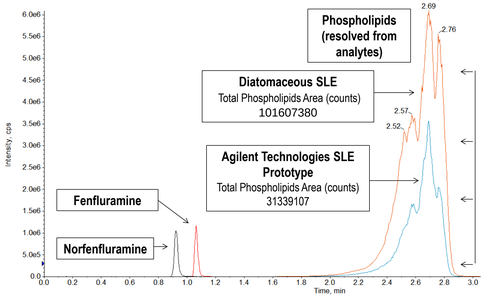


Figure 3. Phospholipids vs. fenfluramine and norfenfluramine in human plasma.

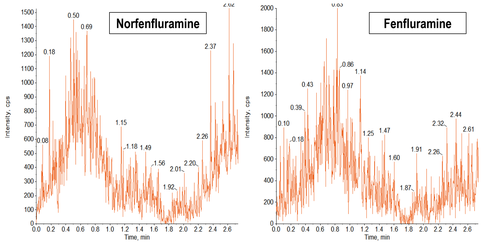


Figure 4. Extracted Blank for fenfluramine and norfenfluramine.

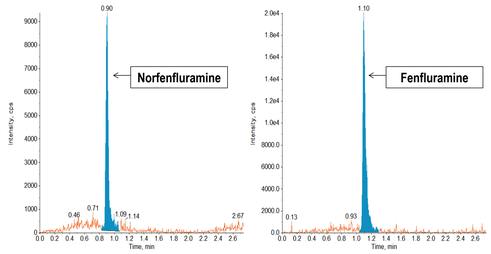


Figure 5. Extracted LOQ (5.00 pg/g) for fenfluramine and norfenfluramine.

Discussion

Following the evaluation of an elution solvent screening panel, the binary composition of ethyl acetate:dichloromethane (1:1) resulted in optimal recovery for both FNN (logP 3.47) and NFNN (logP 2.68) when eluted in a total volume of 800 µL. This low elution volume is advantageous when contrasted to currently available commercial SLE sorbents whose lowest bed mass is 200 mg, and thus require at least 1 mL of elution solvent. In leveraging a lower bed mass (160 mg), a 20% reduction in solvent cost is realized, throughput is increased due to reduced evaporation time and extract may be collected in 1 mL 96-well plates (ideal for sample reconstitution when compared to their 2 mL counterparts). Further, lower bed masses are better aligned with the sample volumes often encountered for preclinical toxicology assessments.

Due to the complex and tedious nature of dissecting cerebellum for all calibrants and QCs, initial experiments were aimed at demonstrating equivalent recovery and matrix effect for FNN and NFNN when extracted from whole brain or cerebellum homogenate. In this manner, whole brain homogenate could be used to quantitate concentrations of FNN and NFNN in cerebellum study samples.

The Agilent SLE prototype furnished recoveries of 80% and 72% for FNN and NFNN, respectively. In contrast, recoveries from diatomaceous SLE were 68% (FNN) and 65% (NFNN). Comparison of phospholipid profiles in homogenate extracts from SLE prototype and diatomaceous substrates revealed ca. 43% greater breakthrough in the latter, indicating the greater trapping efficiency of these suppressors when using the SLE prototype in combination with more highly polar elution solvents.

Intra- and inter-day precision and accuracy batches using the SLE prototype met all acceptance criteria without matrix effect or interference as evaluated from eight individual donors. Curves were linear with an LOQ of 5.00 pg/g, the latter achieved with S/N ratio of 10:1.

CONCLUSIONS

The quantitation of fenfluramine and norfenfluramine from mouse whole brain and cerebellum homogenate using a 160 mg bed mass Agilent SLE prototype proved to be both precise and accurate. This novel low bed mass SLE sorbent retained a significant abundance of phospholipids, thereby providing a cleaner extract and high throughput sample process for low sample volume extractions often needed for preclinical drug quantitation.