WHITE PAPER

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LC Column and SPE Method Solutions for LC-MS/MS Analysis of TDM Drugs and Metabolites

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Introduction

Therapeutic Drug Monitoring (TDM) is an essential practice in clinical settings aimed at optimizing drug therapy, ensuring efficacy, and minimizing toxicity. One of the most powerful analytical techniques used in TDM is Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). This technique has revolutionized the field by providing highly accurate, sensitive, and specific measurements of drug concentrations in biological samples. TDM involves measuring drug levels in blood or other biological fluids at designated intervals to maintain a consistent therapeutic concentration. The primary objectives are to optimize individual dosage regimens, improve therapeutic outcomes, and minimize adverse effects. TDM is particularly crucial for drugs with narrow therapeutic indices, where the margin between therapeutic and toxic doses is minimal.

LC-MS/MS is a powerful analytical technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. LC-MS/MS has become the gold standard for TDM due to its numerous advantages. LC-MS/MS can detect and quantify very low concentrations of drugs and their metabolites, providing highly accurate and specific measurements. The technique can measure a broad range of concentrations, from trace levels to high concentrations, making it suitable for various drugs with different therapeutic ranges. LC-MS/MS can not only simultaneously quantitate multiple drugs and their metabolites in a single run, increasing efficiency and reducing the need for multiple tests, but also offers quick analysis, which is crucial for timely decision-making in clinical research settings. Only small volumes of biological samples are needed, making the process less invasive. In this white paper, we will discuss how SPE and LC-MS/MS can be used to analyze a TDM drug panel of 47 drug analytes from multiple drug classes (**Table 1**). Combined, these methods can be implemented for a screen and confirmation workflow for forensic toxicology.

Table 1. Drugs and Metabolites.

Analyte	Drug Class	Analyte	Drug Class
Selegiline	Antidepressant	Olanzapine	Antipsychotic
Hydroxy bupropion	Antidepressant	Norclozapine	Antipsychotic
Bupropion	Antidepressant	Clozapine	Antipsychotic
Venlafaxine	Antidepressant	9-OH-Risperidone	Antipsychotic
Mirtazapine	Antidepressant	Haloperidol	Antipsychotic
Citalopram	Antidepressant	Risperidone	Antipsychotic
N-Desmethyl-Doxepin	Antidepressant	Promethazine	Antipsychotic
Doxepin	Antidepressant	Quetiapine	Antipsychotic
Trazodone	Antidepressant	Ziprasidone	Antipsychotic
Norfluoxetine	Antidepressant	Dehydroariprazole	Antipsychotic
Fluoxetine	Antidepressant	Chlorpromazine	Antipsychotic
Amoxapine	Antidepressant	Fluphenazine	Antipsychotic
Desipramine	Antidepressant	Ariprazole	Antipsychotic
Imipramine	Antidepressant	Lurasidone	Antipsychotic
Duloxetine	Antidepressant	Pregabalin	Anticonvulsant
Nortriptyline	Antidepressant	Gabapentin	Anticonvulsant
Paroxetine	Antidepressant	Levetiracetam	Anticonvulsant
Amitriptyline	Antidepressant	Lamotrigine	Anticonvulsant
Trimipramine	Antidepressant	Felbamate	Anticonvulsant
N-Desmethyl-Clomipramine	Antidepressant	Lacosemide	Anticonvulsant
Clomipramine	Antidepressant	Zonisamide	Anticonvulsant
Sertraline	Antidepressant	Topiramate	Anticonvulsant
		Oxcarbazapine	Anticonvulsant
		Carbamazepine Epoxide	Anticonvulsant
		Carbamazepine	Anticonvulsant

Analysis of 47 Prescribed Drugs and Metabolites Using a Biphenyl Column and Phospholipid Removal

A crucial step in analyzing these bioanalytical samples is the removal of phospholipids. Phospholipids naturally occur in blood samples and can cause issues when injected into the LC-MS/MS system. They can accumulate on LC columns, leading to a shorter lifespan for the columns. They can also build up in the mass spectrometer, ultimately reducing instrument performance over time. When phospholipids co-elute with analytes of interest, they can affect ionization and cause matrix effects. Serum and plasma samples are often prepared for LC-MS/MS by using protein precipitation or solid phase extraction (SPE). However, protein precipitation alone does not remove phospholipids, and SPE methods require multiple steps, which increase turnaround time and labor costs. To address this, Phree™ phospholipid removal (PLR) can be used as a fast and effective solution to simultaneously remove proteins and phospholipids, resulting in a clean sample suitable for reconstitution and subsequent LC-MS/MS analysis of the target analytes.

As an example, we demonstrate a fast and accurate method for the LC-MS/MS analysis of 47 drug analytes from multiple drug classes (**Table 1**) using a single-step Phree PLR sample preparation to remove proteins and phospholipids. This is combined with a fast LC method using a Kinetex™ 2.6 µm Biphenyl LC column to resolve all target analytes and a SCIEX® QTRAP® 6500+ system for MS/MS analysis. Additionally, the phospholipid content of prepared Phree-extracted samples was compared to protein precipitation alone to assess removal success.

Sample Preparation

A total of 47 TDM compounds were spiked into blank human serum at concentrations of 1 ng/mL, 5 ng/mL, and 50 ng/mL. Then, 100 µL aliquots were added to protein precipitation plates with an oleophobic membrane and a sorbent to selectively remove phospholipids (Phree PLR, Part No.: 8E-S133-TGB). Protein precipitation began upon the addition of 300 µL Acetonitrile, followed by gentle shaking for 5 minutes before elution and collection of the filtrate on a Preston positive pressure manifold. Control samples were prepared at a concentration of 50 ng/mL in blank serum, and 100 µL aliquots were protein precipitated with the addition of 300 µL Acetonitrile in a centrifuge tube. The supernatant was then filtered into autosampler vials through a 0.2 µm filter.

LC Conditions

Column: Kinetex 2.6 µm Biphenyl
Dimensions: 100 x 2.1 mm
Part No.: 00D-4622-AN
Mobile Phase: A: 5 mM Ammonium Formate + 0.1 % Formic Acid in Water
B: 5 mM Ammonium Formate + 0.1 % Formic Acid in Methanol / Acetonitrile (50:50, v/v)

Gradient:

Time (min)	%B
0	30
3	50
5	100
7	100
7.1	10
9.5	10

Flow Rate: 0.4 mL/min
Injection Volume: 2 µL
Temperature: 40 °C
LC System: Shimadzu® LC-30AD
Detection: MS/MS
Detector: SCIEX QTRAP 6500+

MS/MS Conditions

Ion Source: Turbo Spray IonDrive
Polarity: Positive
Source Temperature: 450° C
GS1: 55 psi
GS2: 65 psi
CUR: 35 psi
CAD: 10
IS: 2500 V

Figure 1. Phospholipid Transition 184 → 184 Showing Phospholipid Removal in Phree Extract Compared to Protein Precipitation Only.

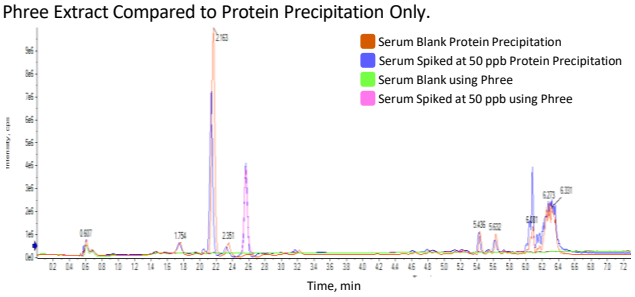
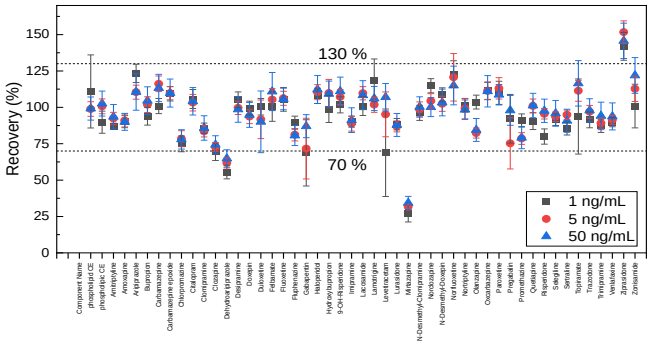


Table 2. Average Concentration and % Recovery of 47 Drug Analytes at 1 ng/mL, 5 ng/mL, and 50 ng/mL Spike Concentrations.

	1 ng/mL		5 ng/mL		50 ng/mL	
Component Name	Average Concentration	% Recovery	Average Concentration	% Recovery	Average Concentration	% Recovery
phospholipid CE	1.1	110.9	4.9	98.8	49.6	99.2
phospholipic CE	0.9	89.8	5.0	100.7	51.3	102.6
Amitriptyline	0.9	87.0	4.6	92.3	46.8	93.6
Amoxapine	0.9	90.2	4.5	90.9	45.0	90.1
Aripiprazole	1.2	123.3	5.5	110.4	55.3	110.6
Bupropion	0.9	93.9	5.1	101.9	52.3	104.6
Carbamazepine	1.0	100.9	5.8	116.2	56.7	113.3
Carbamazepine Epoxide	1.1	109.3	5.5	109.7	54.9	109.8
Chlorpromazine	0.7	74.9	3.9	78.4	39.0	78.0
Citalopram	1.1	105.7	5.2	103.2	52.1	104.2
Clomipramine	0.9	85.6	4.2	83.5	42.9	85.8
Clozapine	0.7	69.5	3.6	72.7	37.2	74.3
Dehydroaripiprazole	0.6	55.5	3.1	61.8	32.4	64.9
Desipramine	1.1	105.3	5.0	99.7	49.3	98.6
Doxepin	1.0	99.5	4.7	93.6	47.4	94.8
Duloxetine	1.0	100.4	4.6	91.8	45.0	90.1
Felbamate	1.0	100.5	5.3	105.4	55.4	110.9
Fluoxetine	1.1	105.5	5.3	106.3	52.9	105.7
Fluphenazine	0.9	89.5	4.1	81.2	40.3	80.7
Gabapentin	0.7	68.7	3.6	71.7	43.5	87.0
Haloperidol	1.1	107.9	5.5	110.4	56.2	112.3
Hydroxybupropion	1.0	98.8	5.5	109.8	54.6	109.3
9-OH-Risperidone	1.0	101.7	5.4	107.1	55.5	111.1
Imipramine	0.9	90.2	4.4	88.8	45.6	91.2
Lacosamide	1.0	100.9	5.5	109.7	54.2	108.4
Lamotrigine	1.2	118.6	5.1	101.8	53.0	106.1
Levetiracetam	0.7	68.8	4.8	95.2	53.5	107.0
Lurasidone	0.9	88.5	4.3	86.9	44.0	87.9
Mirtazapine	0.3	26.9	1.6	31.6	17.2	34.3
N-Desmethyl-Clomipramine	1.0	96.2	4.9	98.6	50.2	100.3
Norclozapine	1.1	114.8	5.2	104.4	50.1	100.2
N-Desmethyl-Doxepin	1.1	108.8	5.1	102.4	51.7	103.3
Norfluoxetine	1.2	122.9	6.0	120.7	57.5	115.0
Nortriptyline	1.0	101.4	5.0	99.2	49.2	98.4
Olanzapine	1.0	103.6	4.1	82.3	42.2	84.5
Oxcarbazepine	1.1	111.3	5.6	111.3	55.5	111.0
Paroxetine	1.1	110.0	5.7	113.1	54.3	108.7
Pregabalin	0.9	92.2	3.8	75.3	49.0	97.9
Promethazine	0.9	91.0	3.9	78.6	39.6	79.2
Quetiapine	0.9	90.5	5.0	101.0	50.6	101.3
Risperidone	0.8	80.0	4.8	95.6	48.8	97.6
Selegiline	0.9	91.8	4.7	93.6	48.0	95.9
Sertraline	0.9	85.4	4.7	94.9	45.4	90.8
Topiramate	0.9	93.7	5.6	111.4	58.2	116.5
Trazodone	0.9	91.4	4.8	97.0	49.0	97.9
Trimipramine	0.9	87.3	4.5	89.1	47.1	94.1
Venlafaxine	0.9	89.9	4.6	91.4	46.9	93.8
Ziprasidone	1.4	141.9	7.6	151.7	72.9	145.7
Zonisamide	1.0	100.9	5.6	112.8	61.0	122.0

Figure 2. Recovery of 47 Drug Analytes Across all Spiked Concentrations.



The Phree™ PLR extraction successfully removed phospholipids from serum samples when compared to only protein precipitation alone (**Figure 1**). This resulted in reduced ion suppression and improved detection of the target analytes. External calibration curves without internal standards were constructed using a regression model of 1/x, showing good linearity with R^2 values ≥ 0.991 for all analytes.

Percent recovery values and average concentrations for all analytes are summarized in **Table 2**. Percent recovery was between 70 % and 130 % for all compounds, except for Dehydroaripiprazole and Mirtazapine, which fell below 70 %. Average concentrations for extraction replicates (n=3) were calculated based on 1 ng/mL, 5 ng/mL, and 50 ng/mL spikes (**Figure 2**). Mirtazapine was the only compound that had consistently low calculated concentrations compared to the amount in the spiked samples. The addition of internal standards and a calibration curve using a response factor would improve concentration accuracy.

The Phree PLR is a single pass through SPE that combines protein precipitation and phospholipid removal that does not retain compounds of interest. Finally, the use of a Kinetex™ 2.6 µm Biphenyl LC column, in conjunction with the sensitivity and robustness of the QTRAP® 6500+ system from SCIEX®, enabled fast sample preparation and analysis for this large panel of analytes.

Screening SPE Sorbents Using a Method Development Plate

Most of the work activity and operating cost in an analytical lab is spent in preparing and processing samples for injection. A simple and structured workflow can save time and cost. SPE provides the maximum cleanliness and sensitivity for LC-MS/MS, however, it has the longest method development time. Knowledge of analyte properties can streamline method development, but sometimes that information is not readily available and large panels of analytes can mean different optimal conditions for different compounds. To develop an efficient and structured approach for sample preparation method development, a Strata-X method development 96-well plate can be utilized. This method development plate is packed with 4 different Polymeric SPE sorbents, allowing for screening of multiple conditions on multiple chemistries to determine best sorbent and starting extraction conditions. Here, a 96-well method development plate was utilized to streamline method development for a panel of 47 prescribed drugs and metabolites: 22 antidepressants, 14 antipsychotics, and 11 anticonvulsants.

The Strata™-X Method Development plate has 4 different polymeric sorbent chemistries: Strata-X, hydrophobic interaction SPE, Strata-X-C, mixed mode strong cation exchange, Strata-X-CW, mixed mode weak cation exchange, and Strata-X-AW, mixed mode weak anion exchange. Three different pre-treatment and elution conditions were screened for each sorbent chemistry:

MD Plate Conditions		Load and Wash	Elution
NN	Neutral load and wash, neutral elution solvent	25 mM Ammonium Acetate, pH 6.9	Methanol / Acetonitrile (1:1, v/v)
AB	Acidic load and wash, basic elution solvent	25 mM Ammonium Formate, pH 4-5	Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v)
BA	Basic load and wash, acidic elution solvent	25 mM Ammonium Bicarbonate, pH 9	1% Formic Acid in Methanol / Acetonitrile (1:1, v/v)

This resulted in 12 SPE method conditions evaluated using a single 96-well plate. The Strata-X Method Development 96-well plate (Part No.: KS0-8209) was set up with the conditions shown in the plate map in **Figure 3**. Spiked human serum was used for this study. Samples were spiked pre- and post-extraction with the appropriate drug class standard mix at a concentration of 1 ng/mL. A general screening (**Table 3**) discussed by drug class in subsequent sections of this white paper was followed.

Figure 3. Plate Map of Method Development Plate.

Strata-X			Strata-X-C			Strata-X-CW			Strata-X-AW		
NN	AB	BA	NN	AB	BA	NN	AB	BA	NN	AB	BA
Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank	Reagent Blank
Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank	Matrix Blank
Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum
Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum	Post-spiked Serum
Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum
Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum
Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum
Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum	Pre-spiked Serum

Table 3. Method Development Plate Extraction Protocol.

Step	Description
Sample Pretreatment:	500 µL human serum was spiked with 1 mg/mL drug category mix and then diluted with the appropriate loading buffer for the specific condition (Neutral, Acidic, Basic).
Condition:	Strata-X SPE Method Development 96-well plate (Part No.: KS0-8209) with 1 mL of Methanol.
Equilibrate:	1 mL Water.
Load:	1 mL of one of the following according to the specific condition: • Neutral Load: 25 mM Ammonium Acetate • Acidic Load: 25 mM Ammonium Formate, pH 4-5 adjusted • Basic Load: 25 mM Ammonium Bicarbonate, pH 9
Wash 1:	1 mL of one of the following according to the specific condition: • Neutral Wash: 25 mM Ammonium Acetate • Acidic Wash: 25 mM Ammonium Formate, pH 4-5 adjusted • Basic Wash: 25 mM Ammonium Bicarbonate, pH 9
Wash 2:	1 mL Methanol / Water (1:1, v/v).
Dry:	5-8 minutes at 20-25 in. Hg.
Elute:	2 aliquots of 300 µL of the following according to the specific condition: • Neutral Elution: Methanol / Acetonitrile (1:1, v/v) • Acidic Elution: Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) • Basic Elution: Methanol / Acetonitrile (1:1, v/v) + 1 % Formic Acid
Dry Down:	15-20 minutes at 40 °C under a gentle stream of Nitrogen.
Reconstitution:	500 µL on initial mobile phase spiked with 5 ng/mL Internal Standard mix.

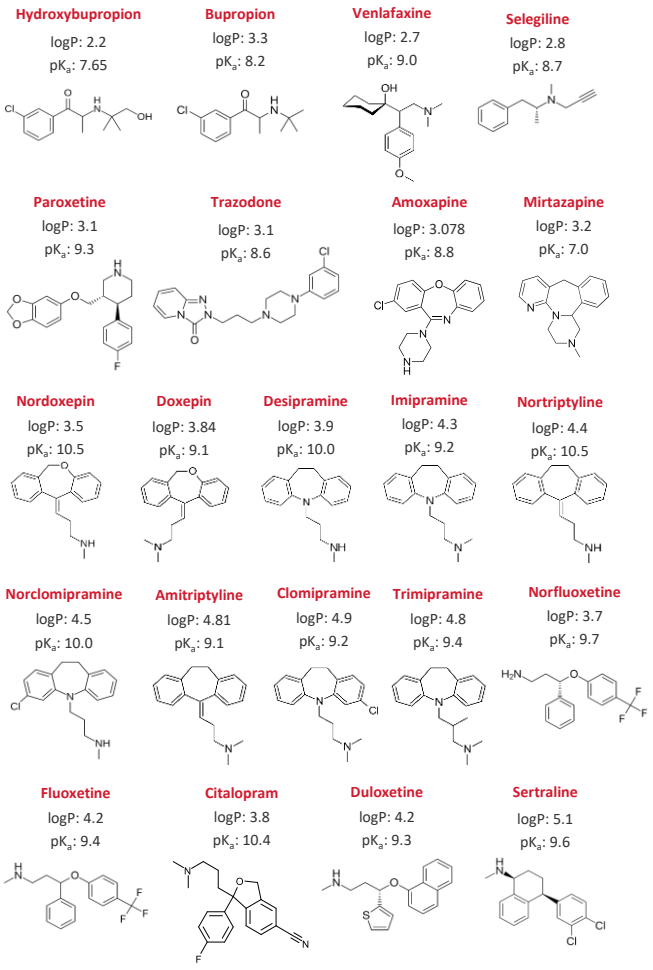
Antidepressants

Antidepressants, such as Selective Serotonin Reuptake Inhibitors (SSRIs) and tricyclic antidepressants (TCAs), exhibit considerable variability in how they are metabolized by different individuals. Genetic factors, liver function, age, and concurrent medications can all influence drug levels. TDM allows for personalized dosing, ensuring that each patient receives an effective yet safe dose. In clinical research, ensuring adherence to the medication regimen is crucial for the validity of study results. TDM can objectively measure adherence, thereby enhancing the reliability of research findings. Antidepressants can have serious side effects, including cardiovascular issues with TCAs or increased suicidal ideation with SSRIs, especially in young adults. Regular monitoring can help identify and mitigate these risks.

Antidepressants are frequently involved in overdose cases, intentional or accidental. TDM can help forensic toxicologists determine whether drug toxicity was a factor in a person's death. In cases of suspected overdose, TDM provides objective data that can be used in court to support or refute claims about the cause of death or impairment. Many individuals taking antidepressants are on multiple medications, increasing the risk of harmful drug interactions. TDM can help identify these interactions postmortem, providing a clearer picture of the deceased's health status and potential causes of death.

As an example, a panel of 22 Antidepressants were analyzed by LC-MS/MS using the Strata™-X Method Development plate that was previously discussed. Based on these results, a final, optimized method was developed using only 10 µL of serum and microelution SPE.

Figure 4. Antidepressant Structures.



LC-MS/MS Analysis and SPE Method Development

The Strata-X-C sorbent under the AB conditions gave the maximum % absolute recovery for the majority of the Antidepressants analyzed using the method development plate (**Table 5**). Please refer to technical note TN-0163 for an in-depth analysis. However, there were two exceptions: Bupropion and Selegiline. Bupropion is a basic analyte with a hydrophobic nature (LogP = 3.2) and only had a % recovery of 35 %. This could be due to Hydrogen bonding and formation of a 5-member ring on its sidechain. This could cause incomplete elution from the Strata-X-C sorbent. Bupropion had higher recovery using Strata-X-CW 95 % or Strata-X 87 %. Selegiline had a % recovery of only 10 %. However, there was good recovery from all other sorbents under BA elution conditions for Selegiline: Strata-X 83 %, Strata-X-CW 86 %, and Strata-X-AW 92 %. This suggests that Selegiline is too retentive on the Strata-X-C sorbent and would require a stronger elution solvent, such as 5 % Ammonium Hydroxide in Methanol / Acetonitrile (1:1, v/v), to completely dislodge the analyte from the sorbent.

To evaluate the chromatography from a matrix-matched sample, 22 Antidepressants were spiked into serum and extracted using the Strata-X Method Development plate before being analyzed on a Luna™ Omega 3 µm Polar C18 and a Kinetex™ 2.6 µm Biphenyl column. The Biphenyl column showed better separation of the analytes. For more in-depth details about the LC-MS/MS analysis, please refer to technical note TN-1356. The mix of Antidepressants showed good peak shape and separation. It also resolved the two critical isobaric pairs: Venlafaxine and Amitriptyline, and Mirtazapine and N-Desmethyl-Doxepin.

This LC-MS/MS method successfully separated the 22 Antidepressant drugs and metabolites, including the two isobaric pairs Venlafaxine and Amitriptyline, and Mirtazapine and N-Desmethyl-Doxepin. The Kinetex 2.6 µm Biphenyl column had a run time of 8 mins and used an easy to prepare mobile phase (Formic Acid in Water and Methanol). This column provided excellent chromatography, and the separation and selectivity required for LC-MS/MS analysis of these TDM analytes.

Microelution SPE and LC-MS/MS Analysis

Microelution SPE requires less solvent for the wash and elution steps. Although the Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent resulted in good recovery for most Antidepressants extracted using the Strata-X-C SPE sorbent from the Strata-X Method Development 96-well plate, it wasn't ideal for direct injection using microelution SPE. These strongly hydrophobic, basic analytes can also be extracted using mixed mode weak cation exchange SPE with Strata-X-CW. Strata-X-CW with a modified elution solvent also had higher recovery for Bupropion and Selegiline compared to Strata-X-C using the method development plate. Therefore, a Strata-X-CW 2 mg/well 96-well microelution plate was employed for extraction of Antidepressants in serum under AB condition (acid load and basic elution). The optimized microelution method aimed toward higher recovery of analytes and direct injection of extracted samples that bypasses the dry-down and reconstitution steps. Please refer to technical note TN-0165 for more details.

Comparison of results from the 30 mg/well Method Development plate using Strata-X-C and Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent to the results using the 2 mg/well microluetion Strata-X-CW plate and the 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40, v/v) elution solvent demonstrated higher recovery with the microleution SPE conditions for all analytes except Trazodone and Duloxetine. Recovery for those two compounds was still greater than 80 % using microelution SPE (**in Green**, **Table 4**). The optimized microelution method is shown in Table 5, compared to the Method Development plate conditions that were previously established with a representative chromatogram of extracted samples in **Figure 5**.

LC Conditions

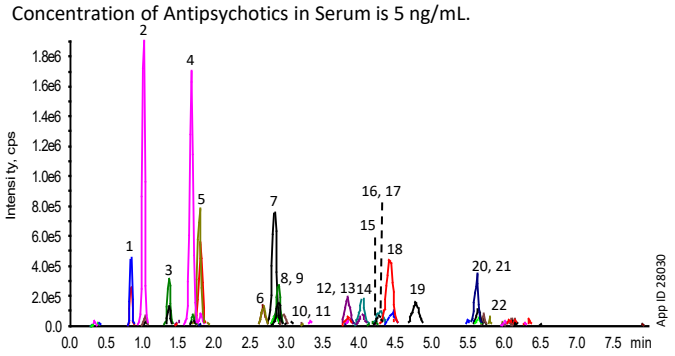
Column: Kinetex 2.6 µm Biphenyl
Dimensions: 50 x 3.0 mm
Part No.: 00B-4622-Y0
Mobile Phase: A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Methanol

Gradient:

Time (min)	% B
0	20
1	40
2	80
3	95
3.5	95
3.51	20
5	20

Flow Rate: 0.7 mL/min
Injection Volume: 5 µL
Temperature: 40 °C
LC System: Agilent® 1260 Infinity
Detection: MS/MS
Detector: SCIEX® 6500 Triple Quad™

Figure 5. Analysis of Antidepressants Extracted from Serum Using Strata™-X-CW Microelution Plate on a Kinetex™ 2.6 µm Biphenyl Column. Spiked Concentration of Antipsychotics in Serum is 5 ng/mL.



Peak No.	Analyte	Retention Time (min)	Peak No.	Analyte	Retention Time (min)
1	Selegiline	0.84	12	Amoxapine	3.8
2	Hydroxybupropion	1.1	13	Desipramine	3.9
3	Bupropion	1.4	14	Imipramine	4.1
4	Venlafaxine	1.7	15	Duloxetine	4.2
5	Mirtazapine	1.8	16	Nortriptyline	4.3
6	Citalopram	2.6	17	Paroxetine	4.3
7	N-Desmethyl-Doxepin	2.8	18	Amitriptyline	4.5
8	Doxepin	2.9	19	Trimipramine	4.8
9	Trazodone	2.9	20	N-Desmethyl-Clomipramine	5.6
10	Norfluoxetine	3.3	21	Clomipramine	5.6
11	Fluoxetine	3.3	22	Sertraline	5.7

Percent recovery of all analytes at three spiked concentrations, N=4, (10 ng/mL, 40 ng/mL, and 400 ng/mL) ranged from 72-127 % with % CV <24 % for all analytes (≤20 % consistency observed within the 3 concentration levels tested), except for Duloxetine, % CV = 34 % (**Table 4**).

Matrix effect ranged from 34 % suppression and 20 % enhancement for the 3 concentrations evaluated except Duloxetine at 40 ng/mL, which was 54 %. Process efficiency (PE) was 71-119 %, except Duloxetine at 10 ng/mL, which was 42 %. Calibration curves overa a 100-fold dynamic range (5 ng/mL to 500 ng/mL) with 1/x weighting demonstrated excellent linearity with R^2 values ≥0.993 for all target compounds (**Figure 6**, **Table 6**), from the least hydrophobic (Hydroxybupropion, logP of 2.2) to the most hydrophobic (Sertraline, logP of 5.1).

Precision and accuracy were 0.5-17.8 % and 93-115 %, respectively for all analytes at all three concentrations evaluated. These results meet the generally accepted criteria of ±20 % for precision and 85-115 % for accuracy, except for Duloxetine where precision was 25 % at 10 ng/mL.

Table 4. Recovery Comparison of Antidepressants Extracted from Serum Using the Strata-X-C SPE Sorbent of the Strata-X Method Development 30 mg/well 96-Well Plate and the Strata-X-CW Microelution 2 mg/well 96-Well Plate.

	Strata-X-C SPE Sorbent of the Strata-X Method Development 30 mg/well 96-Well Plate *Spiked conc. of serum = 1 ng/mL		Strata-X-CW Microelution 2 mg/well 96-Well Plate *Spiked conc. of serum = 10 ng/mL	
	Elution Solvent: Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v)		Elution Solvent: 5 % Ammonium Hydroxide in in Acetonitrile / Methanol (60:40, v/v)	
Analyte	% Recovery	% CV (N = 4)	% Recovery	% CV (N = 4)
Selegiline	10	65	**76**	**11.4**
Hydroxybupropion	81	4.8	**102**	**5.8**
Bupropion	35	30.5	**98**	**5.7**
Venlafaxine	84	10.9	**91**	**3.7**
Mirtazapine	79	4.2	**91**	**2.9**
Citaprolam	81	5.2	**109**	**3.8**
N-Desmethyl-Doxepin	80	5.5	**89**	**2.1**
Doxepin	83	1.9	**101**	**5.9**
Trazodone	82	7.3	**99**	**1.6**
Norfluoxetine	82	10.1	**99**	**11.4**
Fluoxetine	78	7.5	**79**	**5.2**
Amoxapine	79	6	**98**	**3.9**
Desipramine	77	7.1	**77**	**3.2**
Imipramine	78	7.8	**95**	**4.2**
Duloxetine	112	14.5	**86**	**25.8**
Nortriptyline	78	6.4	**85**	**5.3**
Paroxetine	79	3.1	**86**	**7.2**
Amitriptyline	78	2.8	**90**	**3.2**
Trimipramine	74	1.6	**83**	**2.9**
N-Desmethyl-Clomipramine	73	5.5	**95**	**2.7**
Clomipramine	80	4.8	**87**	**3.7**
Sertraline	94	10.9	**81**	**2.6**

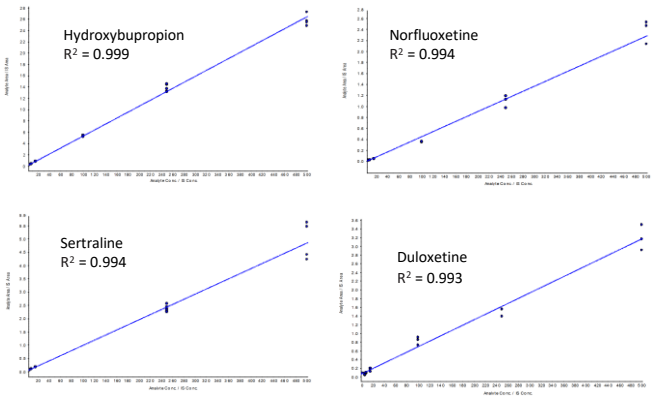
Table 5. SPE Method Comparison of Extracting Antidepressants from Serum Using the Strata-X-C SPE Sorbent of the Strata-X Method Development 30 mg/well 96-Well Plate (Left) and the Strata-X-CW Microelution 2 mg/well 96-Well Plate (Right).

Step	Description	Step	Description
Sample Pretreatment:	500 µL human serum was spiked with Antidepressants standard mix at a concentration of 1 ng/mL (except Duloxetine at 6 ng/mL) and then diluted with 1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted.	**Sample Pretreatment:**	10 µL human serum was spiked with Antidepressants standard mix and internal standards and then diluted with 200 µL of 25 mM Ammonium Formate, pH ~3.5 adjusted.
Condition:	Strata-X Method Development 96-well plate, 30 mg/well (Part No.: KS0-8209) with 1 mL of Methanol.	**Condition:**	Strata-X-CW Microelution 96-well plate, 2 mg/well (Part No.: 8M-S035-4GA) with 200 µL of Methanol.
Equilibrate:	1 mL Water.	**Equilibrate:**	200 µL Water.
Load:	About 1.5 mL of pre-treated sample.	**Load:**	200 µL diluted pre-treated sample.
Wash 1:	1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted.	**Wash 1:**	200 µL of Acidic Buffer 25 mM Ammonium Formate, pH ~3.5 adjusted
Wash 2:	1 mL Methanol / Water (1:1, v/v).	**Wash 2:**	200 µL Methanol / Water (1:1, v/v).
Dry:	5-8 minutes at 20-25 in. Hg.	**Dry:**	1 minute at 20-25 in. Hg.
Elute:	2 aliquots of 300 µL of Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v).	**Elute:**	2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40).
Dry Down:	15-20 minutes at 40 °C under a gentle stream of Nitrogen. Time ≅ 25 min Total Dry Time ≅ 35 min	**Dry Down:**	Bypass. Total Dry Time ≅ 1 min
Reconstitution:	500 µL of initial mobile phase spiked with 5 ng/mL Internal Standard mix (Venlafaxine-D_6, Amitriptyline-D_3, N-Desmethyl-Doxepin-D_3, Mirtazepine-D_3, Clomipramine-D_3, Hydroxybuprpion-D_6, Duloxetin-D_3). Time ≅ 1 min	**Reconstitution:**	Bypass.
		Dilute:	Dilute with 200 µL mobile phase A (0.1 % Formic Acid in Water) before injection.
Total Evaporation and Reconstitution Time:	≅ 36 min/plate	**Total Evaporation and Reconstitution Time:**	≅ 1 min/plate
Total Reagent Volume:	6.1 mL/well ; 585.6 mL/plate	**Total Reagent Volume:**	1.3 mL/well ; 124.8 mL/well

Table 6. Method Performance and Qualification Data Utilizing the Finalized Method for Extracted Antidepressants from Serum Using Strata™-X-CW Microelution Plate.

Analyte	Concentration (ng/mL)	% Recovery	% CV	% Matrix Effect	PE	Precision	Accuracy	Linear regression R^2	Regression Equation
Selegiline	10	76	11.4	102	74	11.3	92	0.995	y =0.0221 x + 0.0289
	40	72	14.3	66	71	14.6	97		
	400	99	12.2	71	71	9.9	96		
Hydroxybupropion	10	102	5.8	102	109	7.4	103	0.999	y = 0.0527 x + 0.0618
	40	108	5.2	105	102	5.3	102		
	400	95	6.4	96	87	4.6	96		
Bupropion	10	98	5.7	98	85	7.5	95	0.998	y = 0.0292 x + 0.152
	40	101	8.4	98	80	8.5	97		
	400	97	3.7	95	76	6.5	94		
Venlafaxine	10	91	3.7	97	95	3.9	97	0.999	y = 0.00624 x + 0.00511
	40	97	4.7	96	97	3.7	101		
	400	98	5	87	86	0.5	92		
Mirtazapine	10	91	2.9	112	106	3.2	99	0.997	y = 0.134 x + 0.131
	40	101	2.6	101	109	2.7	107		
	400	98	6.1	91	91	5.5	92		
Citalopram	10	109	3.8	109	102	4	101	0.997	y = 0.0281 x + 0.041
	40	127	5.6	120	111	1	106		
	400	106	7.5	107	89	11.8	94		
N-Desmethyl-Doxepin	10	89	2.1	89	89	2.4	101	0.999	y = 0.0184 x + 0.228
	40	108	3.8	110	95	3.9	105		
	400	103	8.3	103	92	7.4	101		
Doxepin	10	101	5.9	101	101	12.1	109	0.997	y = 0.0171 x + 0.0173
	40	104	3.6	99	93	13.9	109		
	400	102	3.7	102	91	6.7	90		
Trazodone	10	99	1.6	113	100	8.1	98	0.996	y = 0.0561 x + 0.13
	40	115	5.8	115	115	4.5	109		
	400	112	4	97	96	5.5	95		
Norfluoxetine	10	99	11.4	84	99	9.3	101	0.994	y = 0.00458 x + 0.0084
	40	82	13.1	84	85	11	83		
	400	102	24.4	92	79	17.8	98		
Fluoxetine	10	79	5.2	79	104	4.2	102	0.997	y = 0.00722 x + 0.00232
	40	102	9.7	104	112	9.7	101		
	400	116	16.1	117	119	15	115		
Amoxapine	10	98	3.9	98	99	5	106	0.995	y = 0.0611 x + 0.115
	40	110	6.1	117	119	3.9	110		
	400	91	15.8	91	86	6.9	88		
Desipramine	10	77	3.2	77	93	3.6	106	0.998	y = 0.0185 x + 0.035
	40	87	1.8	90	100	3.1	109		
	400	90	8.8	89	93	10.3	97		
Imipramine	10	95	4.2	95	111	6.5	106	0.997	y = 0.0551 x + 0.124
	40	106	3.9	111	106	4.1	110		
	400	98	8	97	88	6.6	92		
Duloxetine	10	86	25.8	74	42	25	91	0.993	y = 0.00619 x + 0.0753
	40	91	33.8	54	50	12.8	104		
	400	104	20.6	78	71	20	114		
Nortriptyline	10	85	5.3	82	97	6.2	107	0.998	y = 0.0363 x + 0.0644
	40	88	2.9	91	98	3.1	107		
	400	107	12.1	102	97	8.8	93		
Paroxetine	10	86	7.2	85	119	5.9	111	0.998	y = 0.0551 x + 0.124
	40	96	7.6	98	112	1.8	108		
	400	96	14.7	92	115	14.7	93		
Amitriptyline	10	90	3.2	90	96	4	101	0.998	y = 0.0304 x + 0.082
	40	98	1	101	85	0.9	106		
	400	103	12.1	99	81	7.3	104		
Trimipramine	10	83	2.9	95	83	4.1	88	0.996	y = 0.0977 x + 0.35
	40	87	9.2	87	86	7.9	106		
	400	88	11.3	91	88	7.6	85		
N-Desmethyl-Clomipramine	10	95	2.7	70	87	3.2	103	0.997	y = 0.0355 x + 0.067
	40	107	5.7	82	107	6.1	112		
	400	100	19	100	103	15.2	95		
Clomipramine	10	87	3.7	87	85	5.4	98	0.997	y = 0.047 x + 0.166
	40	99	2.8	102	92	3.1	112		
	400	104	13.5	101	84	10.5	101		
Sertraline	10	81	2.6	84	78	3.3	104	0.994	y = 0.00964 x + 0.0288
	40	80	6.4	80	86	1.4	110		
	400	99	19.1	94	95	3.2	86		

Figure 6. Calibration Curves for Four Selected Antidepressants Over 100-Fold Dynamic Range (5 ng/mL to 500 ng/mL).



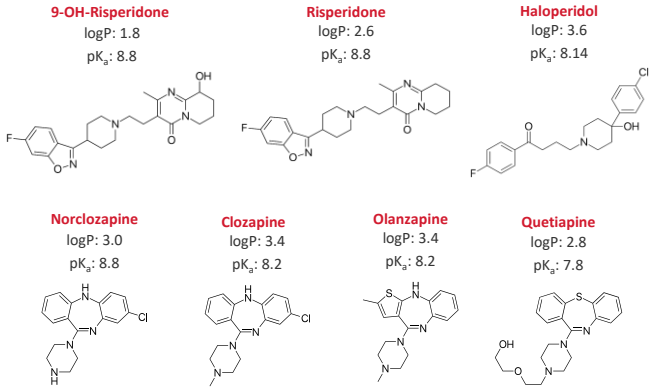
Antipsychotics

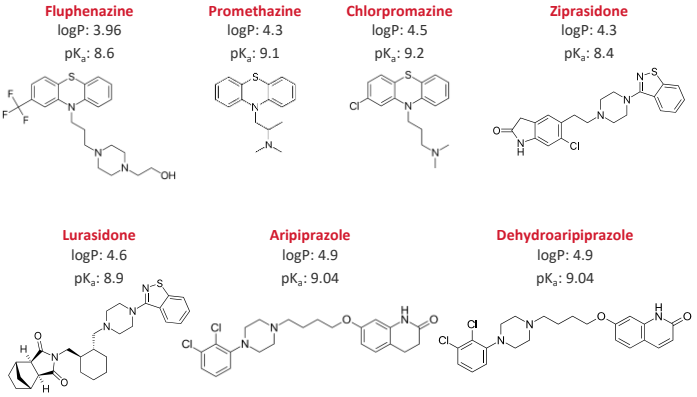
Antipsychotics, such as clozapine and risperidone, have a narrow therapeutic index. The difference between a therapeutic dose and a toxic dose is small, making TDM essential for safe and effective treatment. Antipsychotics can cause severe side effects, including tardive dyskinesia, metabolic syndrome, and agranulocytosis. Monitoring drug levels helps in early detection and management of these side effects. Different users metabolize antipsychotics at different rates due to genetic polymorphisms in enzymes like CYP2D6. TDM allows researchers to adjust doses to achieve optimal therapeutic levels, improving the efficacy of the treatment.

Overdose with antipsychotics can lead to severe toxicity, including cardiac arrhythmias and neuroleptic malignant syndrome. TDM can help determine if an overdose was the cause of death. Antipsychotics can significantly alter behavior. In forensic cases involving violent behavior or accidents, TDM can provide insights into whether the drug levels were within the therapeutic range or if they contributed to the incident. In cases of substance abuse, TDM can help distinguish between therapeutic use and misuse of antipsychotic drugs, providing critical information for legal and medical investigations.

For example, a panel of 14 Antipsychotics were analyzed by LC-MS/MS using the Strata™-X Method Development plate that was previously discussed. Based on these results, a final, optimized method was developed using only 10 µL of serum and microelution SPE.

Figure 7. Antipsychotic Structures.





LC-MS/MS Analysis and SPE Method Development

The Strata-X-CW sorbent under the AB conditions gave the maximum % absolute recovery for most of the Antipsychotics analyzed (**Table 7**). Please refer to technical note TN-0163 for an in-depth analysis. However, there was a single exception: Chlorpromazine. Chlorpromazine is a basic analyte with a hydrophobic nature (LogP = 4.5) and only had a % recovery of 59 % due to strong retention on the Strata-X-CW sorbent. There was much better recovery using the neutral Strata-X sorbent under the same AB elution conditions. A stronger elution solvent, such as 5 % Ammonium Hydroxide in Methanol / Acetonitrile (1:1, v/v), would aid in increasing the recovery of Chlorpromazine. The best sample preparation protocol for Antipsychotics on the Method Development plate using Strata-X-CW is shown in **Table 8**.

To evaluate the chromatography from a matrix-matched sample, 14 antipsychotics were spiked into serum and extracted using the Strata-X Method Development plate before being being analyzed on a Luna™ Omega 3 µm Polar C18 and a Kinetex™ 2.6 µm Biphenyl column. The Biphenyl column showed better separation of the analytes. For more in-depth details about the LC-MS/MS analysis, please refer to technical note TN-1355.

Microelution SPE and LC-MS/MS Analysis

Microelution SPE requires less solvent for the wash and elution steps. Although the Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent resulted in good recovery for Antipsychotics extracted using the Strata-X-CW SPE sorbent from the Strata-X Method Development 96-well plate, it wasn't ideal for direct injection using microelution SPE. A number of different elution solvents were tested and 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40, v/v) elution was found to be more effective than 5 % Ammonium Hydroxide in Methanol by dislodging the more hydrophobic, late eluting analytes. Therefore, a Strata-X-CW 2 mg/well 96-well microelution plate was employed for extraction of Antidepressants in serum under AB condition (acid load and basic elution). The optimized microelution method aimed toward higher recovery of analytes and direct injection of extracted samples that bypasses the dry-down and reconstitution steps. Please refer to technical note TN-0164 for more details.

Comparison of results from the 30 mg/well Method Development plate using Strata-X-C and Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent to the results using the 2 mg/well microluetion Strata-X-CW plate and the 5 % Ammonium Hydroxide in in Acetonitrile / Methanol (60:40, v/v), resulted in higher (blue) recovery for 50 % of the panel and lower (red) for other 50 %, compared to the Strata-X-CW sorbent of the Strata-X Method Development 96-well plate with the Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent (**Table 7**).

The optimized microelution method is shown in Table 8, compared to the Method Development plate conditions that were previously established with a representative chromatogram of extracted samples in **Figure 8**.

LC Conditions

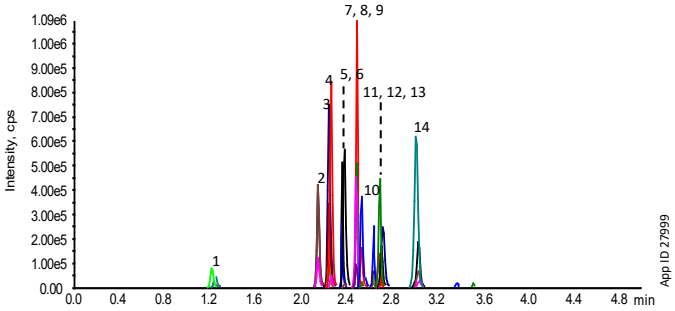
Column: Kinetex™ 2.6 µm Biphenyl
Dimensions: 50 x 3.0 mm
Part No.: 00B-4622-Y0
Mobile Phase: A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Methanol

Gradient:

Time (min)	% B
0	20
1	40
2	80
3	95
3.5	95
3.51	20
5	20

Flow Rate: 0.7 mL/min
Injection Volume: 5 µL
Temperature: 40 °C
LC System: Agilent® 1260 Infinity
Detection: MS/MS
Detector: SCIEX® 6500 Triple Quad™

Figure 8. Analysis of Antipsychotics Extracted from Serum Using Strata™-X-CW Microelution Plate on a Kinetex 2.6 µm Biphenyl Column. Spiked Concentration of Antipsychotics in Serum is 5 ng/mL.



Peak No.	Analyte	Retention Time (min)	Peak No.	Analyte	Retention Time (min)
1	Olanzapine	1.25	8	Quetiapine	2.5
2	Norclozapine	2.14	9	Ziprasidone	2.5
3	Clozapine	2.24	10	Dehydroariprazole	2.6
4	9-OH-Risperidone	2.3	11	Chlorpromazine	2.7
5	Haloperidol	2.4	12	Fluphenazine	2.7
6	Risperidone	2.4	13	Ariprazole	2.7
7	Promethazine	2.53	14	Lurasidone	3.1

Table 7. Recovery Comparison of Antipsychotics Extracted from Serum Using the Strata-X-C SPE Sorbent of the Strata-X Method Development 30 mg/well 96-Well Plate and the Strata-X-CW Microelution 2 mg/well 96-Well Plate.

	Strata-X-CW SPE Sorbent of the Strata-X Method Development 30 mg/well 96-Well Plate *Spiked conc. of serum = 1 ng/mL		Strata-X-CW Microelution 2 mg/well 96-Well Plate *Spiked conc. of serum = 10 ng/mL	
	Elution Solvent: Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v)		Elution Solvent: 5 % Ammonium Hydroxide in in Acetonitrile / Methanol (60:40, v/v)	
Analyte	% Recovery	% CV (N = 4)	% Recovery	% CV (N = 4)
Olanzapine	80	8.9	**58**	**6.8**
Norclozapine	90	10.3	**79**	**11.6**
Clozapine	91	5.5	**121**	**6.1**
9-OH-Risperidone	97	12.3	**103**	**7.1**
Haloperidol	91	1.5	**95**	**12.4**
Risperidone	95	8.4	**109**	**10.5**
Promethazine	97	14.4	**70**	**9.3**
Quetiapine	91	14.5	**70**	**9.3**
Ziprasidone	85	7.9	**88**	**6.8**
Dehydroariprazole	97	9.2	**74**	**12.8**
Chlorpromazine	59	15.8	**70**	**10.6**
Fluphenazine	95	22.6	**74**	**16**
Aripiprazole	85	2.9	**101**	**5.3**
Lurasidone	95	9.2	**74**	**9.3**

The process efficiency (PE) for most of the analytes tended to increase as the concentration increased over the 3 concentrations evaluated (**Table 9**). Matrix effect at all three concentrations for all drug analytes ranged from 34 % to 122 %. Matrix effect generally improved with increasing concentration, with values of 80 % to 103 % for all analytes at 400 ng/mL, except Olanzapine and Lurasidone (Table 9). Calibration curves over a 100-fold dynamic range (5 ng/mL to 500 ng/mL) with 1/x weighting demonstrated excellent linearity with R^2 values ≥0.992 for all target compounds, even those that had lower recovery or exhibited increased ion suppression or enhancement and lower process efficiency (**Table 9**, **Figure 9**).

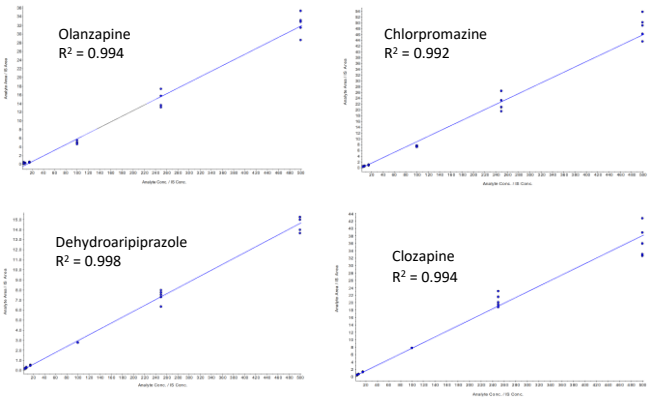
Table 8. SPE Method Comparison of Extracting Antipsychotics from Serum Using the Strata-X-CW SPE Sorbent of the Strata-X Method Development 30 mg/well 96-Well Plate (Left) and the Strata-X-CW Microelution 2 mg/well 96-Well Plate (Right).

Step	Description	Step	Description
Sample Pretreatment:	500 µL human serum was spiked with Antipsychotic standard mix at a concentration of 1 ng/mL (except Fluphenazine at 3 ng/mL) and then diluted with 1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted.	**Sample Pretreatment:**	10 µL human serum was spiked with Antipsychotics standard mix and internal standards and then diluted with 200 µL of 25 mM Ammonium Formate, pH ~3.5 adjusted.
Condition:	Strata-X Method Development 96-well plate, 30 mg/well (Part No.: KS0-8209) with 1 mL of Methanol.	**Condition:**	Strata-X-CW Microelution 96-well plate, 2 mg/well (Part No.: 8M-S035-4GA) with 200 µL of Methanol.
Equilibrate:	1 mL Water.	**Equilibrate:**	200 µL Water.
Load:	About 1.5 mL of pre-treated sample.	**Load:**	200 µL diluted pre-treated sample.
Wash 1:	1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted.	**Wash 1:**	200 µL of Acidic Buffer 25 mM Ammonium Formate, pH ~3.5 adjusted
Wash 2:	1 mL Methanol / Water (1:1, v/v).	**Wash 2:**	200 µL Methanol / Water (1:1, v/v).
Dry:	5-8 minutes at 20-25 in. Hg.	**Dry:**	1 minute at 20-25 in. Hg.
Elute:	2 aliquots of 300 µL of Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v).	**Elute:**	2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40).
Dry Down:	15-20 minutes at 40 °C under a gentle stream of Nitrogen. Time ≅ 25 min Total Dry Time ≅ 35 min	**Dry Down:**	Bypass. Total Dry Time ≅ 1 min
Reconstitution:	500 µL on initial mobile phase spiked with 5 ng/mL Internal Standard mix (Lurasidone-D_8, Ariprazole-D_8, Fluphenazine-D_8, Olanzapine-D_8). Time ≅ 1 min	**Reconstitution:**	Bypass.
		Dilute:	Dilute with 200 µL mobile phase A (0.1 % Formic Acid in Water) before injection.
Total Evaporation and Reconstitution Time:	≅ 36 min/plate	**Total Evaporation and Reconstitution Time:**	≅ 1 min/plate
Total Reagent Volume:	6.1 mL/well ; 585.6 mL/plate	**Total Reagent Volume:**	1.3 mL/well ; 124.8 mL/well

Table 9. Method Performance and Qualification Data Utilizing the Finalized Method for Extracted Antipsychotics from Serum Using Strata™-X-CW Microelution Plate.

Analyte	Concentration (ng/mL)	% Recovery	% CV	% Matrix Effect	PE	Precision	Accuracy	Linear regression R^2	Regression Equation
Olanzapine	10	58	6.8	58	43	15	90	0.994	y = 0.0648 x + -0.586
	40	46	9.4	53	23	7.8	89		
	400	40	19.2	34	29	9.2	85		
Norclozapine	10	79	11.6	79	40	11	98	0.995	y = 0.188 x + 0.244
	40	92	9.4	94	54	9.7	100		
	400	76	14.3	102	83	10.7	85		
Clozapine	10	121	6.1	122	87	5.2	101	0.994	y = 0.076 x + 0.0969
	40	122	3.7	116	116	6.4	99		
	400	116	5.3	99	111	6.7	99		
9-OH-Risperidone	10	103	7.1	102	75	6.3	102	0.995	y = 0.293 x + 0.892
	40	120	6.8	107	94	7.2	115		
	400	97	13	103	111	12.5	88		
Haloperidol	10	95	12.4	94	61	15	101	0.994	y = 0.431 x + 0.995
	40	110	6.7	105	78	7.1	109		
	400	91	13.7	103	97	8.8	85		
Risperidone	10	109	10.5	62	86	4.7	85	0.995	y = 0.206 x + 1.51
	40	115	8.2	78	78	8.5	105		
	400	100	15.4	95	95	13	88		
Promethazine	10	70	9.3	77	60	8.2	88	0.997	y = 0.202 x + -0.0321
	40	78	6.0	86	82	2.4	85		
	400	62	11.4	102	73	11	84		
Quetiapine	10	70	9.3	94	59	12.3	102	0.992	y = 0.432 x + 1.2
	40	78	6	105	67	4.9	111		
	400	62	11.4	103	81	9.5	85		
Ziprasidone	10	88	6.8	83	42	9.4	104	0.995	y = 0.197 x + 0.35
	40	82	7.2	82	64	7.5	109		
	400	75	14.4	80	72	7	87		
Dehydroaripiprazole	10	74	12.8	74	61	14	99	0.998	y = 0.0292 x + 0.0292
	40	79	8.8	89	99	9.1	109		
	400	71	15.4	101	92	8.9	92		
Chlorpromazine	10	70	10.6	63	108	5	88	0.992	y = 0.0921 x + -0.167
	40	65	6.2	76	98	4.7	85		
	400	50	11.7	100	60	2.6	90		
Fluphenazine	10	74	16	74	62	15.9	89	0.993	y = 0.00822 x + -0.000277
	40	78	9.5	88	87	9.5	89		
	400	68	12.9	102	83	9.7	85		
Aripiprazole	10	101	5.3	101	62	5.2	101	0.995	y = 0.0167 x + -0.00437
	40	112	6.4	106	112	6.4	99		
	400	93	5	97	115	6.7	99		
Lurasidone	10	74	9.3	60	75	12.9	113	0.998	y = 0.0972 x + 0.2
	40	99	6.7	63	107	6.9	110		
	400	82	11.9	56	82	9.8	90		

Figure 9. Calibration Curves for Four Selected Antipsychotics Over 100-Fold Dynamic Range (5 ng/mL to 500 ng/mL).



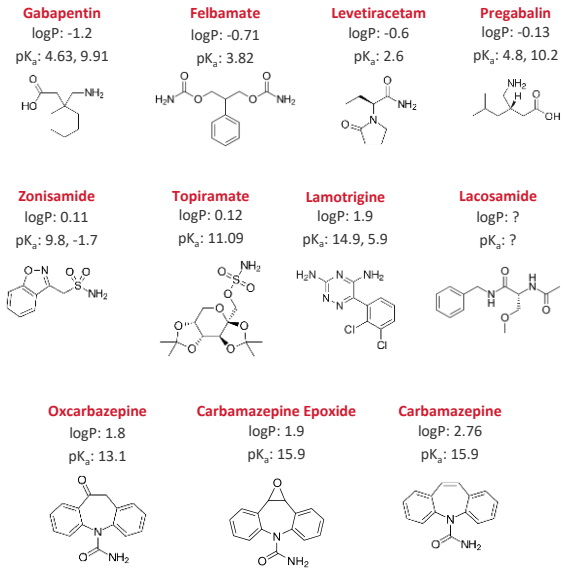
Anticonvulsants

Anticonvulsants like Phenytoin, Valproate, and Carbamazepine are used to control seizures. Maintaining therapeutic levels is crucial for preventing both breakthrough seizures and toxic side effects. TDM helps in achieving this balance. The pharmacokinetics of anticonvulsants can be complex, with factors such as age, liver function, and concurrent medications affecting drug levels. TDM allows for individualized dosing, ensuring effective seizure control with minimal side effects. Many anticonvulsants are enzyme inducers or inhibitors, affecting the metabolism of other drugs. TDM helps in understanding these interactions, allowing for safer and more effective use of multiple medications.

In cases of unexplained deaths, TDM can help determine if anticonvulsant toxicity was a factor. For example, high levels of phenytoin can cause severe central nervous system depression and cardiac arrhythmias, potentially leading to death. Non-adherence to anticonvulsant therapy can result in uncontrolled seizures, which can be fatal. TDM can help forensic toxicologists understand if non-adherence played a role in the cause of death. Anticonvulsants can have a narrow therapeutic index, and overdose can be fatal. TDM provides precise measurements of drug levels, helping in the investigation of suspected overdose cases.

A panel of 11 Anticonvulsants were analyzed by LC-MS/MS using the Strata™-X Method Development plate that was previously discussed. Based on these results, a final, optimized method was developed using only 10 µL of serum and microelution SPE.

Figure 10. Anticonvulsants Structures.



LC-MS/MS Analysis and SPE Method Development

The panel of Anticonvulsants analytes is complicated and diverse, composed of Zwitterions, sulfonamides, and neutral, acidic and basic compounds. There is a wide range of pK_a values and polarity in this drug class, which makes developing a single method challenging. The best recovery for the majority of the analytes used the Strata-X-CW sorbent under AB conditions, however there were several deviations. For more detailed information, please refer to technical note TN-0163. Lacosamide, Felbamate, and Levetiracetam are neutral and very polar compounds with negative LogP values. These compounds began eluting during the 50 % Methanol wash, lowering their overall recovery. Gabapentin and Pregabalin are Zwitterions, and both had about 50 % recovery on both cation exchange phases. A stronger elution solvent may help release the Zwitterions from the sorbent. Oxcarbazepine had 37 % recovery from Strata-X-CW under AB conditions but increased to 71 % under BA conditions. Zonisamide and Topiramate are neutral and relatively polar sulfonamides and had roughly 25-26 % recovery from the Strata-X-CW sorbent under AB conditions. Their recovery improved to 47 % and 83 % on Strata-X under NN conditions. This suggests an incomplete elution so a stronger elution solvent, such as 5 % Ammonium Hydroxide in Methanol / Acetonitrile (1:1, v/v), should improve recovery from the weak exchange (Strata-X-CW) sorbent. Based on these results, further SPE method development was required for the Anticonvulsants.

To evaluate the chromatography from a matrix-matched sample, 11 Anticonvulsants were spiked into serum and extracted using the Strata-X Method Development Plate before being analyzed on a Kinetex™ 2.6 µm Biphenyl column and a Luna™ Omega 3 µm Polar C18 column. The Polar C18 column showed better separation of the analytes. For more in-depth details about the LC-MS/MS analysis, please refer to technical note TN-1357. The mix of Anticonvulsants analytes were separated with good peak shape and demonstrated great selectivity towards the separation of the critical isobaric pair of Oxcarbazapine and Carbazepine Epoxide.

The developed LC-MS/MS method utilizing the Luna Omega 3 µm Polar C18 column has been found to be the optimal chromatography conditions for analysis of the TDM panel of Anticonvulsants. The Method Development plate failed to identify a single optimal protocol for extraction of the Anticonvulsants from serum. Further work was needed to develop an SPE method.

Microelution SPE and LC-MS/MS Analysis

Microelution SPE requires less solvent for the wash and elution steps. A Strata-X-CW 2 mg/well 96-well microelution plate was employed for extraction of Anticonvulsants in serum under AB conditions (acidic load and basic elution). The optimized microelution method aimed toward higher recovery of analytes and direct injection of extracted samples that bypasses the dry-down and reconstitution steps. Please refer to technical note TN-0166 for more details. Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent using the Strata-X-C SPE sorbent from the Strata-X Method Development 96-well plate gave poor recovery for multiple drug analytes and wasn't ideal for direct injection of the extracted samples using microelution SPE. Further method development demonstrated that Strata-X-CW and Methanol treated with 5 % Ammonium Hydroxide and showed improved analyte recovery (by 70-80 %) especially for Zwitterionic compounds. As a protic solvent, Methanol was a stronger solvent for polar analytes which increased solubility and resulted in complete elution of Pregabalin and Gabapentin from the weak cation exchange SPE sorbent.

Comparison of results from the 30 mg/well Method Development plate using Strata-X-C and Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent to the results using the 2 mg/well microluetion Strata-X-CW plate and the 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40, v/v) elution solvent demonstrated higher recovery with the microleution SPE conditions for all analytes except Levetiracetam. Recovery for Levetiracetam was <25 % on both plates (**in Red**, **Table 10**). The optimized microelution method is shown in **Table 11**, compared to the Method Development plate conditions that were previously established with a representative chromatogram of extracted samples in **Figure 11**.

Percent recovery for the optimized microelution method at 10 ng/mL was between 80-124 % (Table 10) except the polar compounds Topiramate (55 %) and Levetiracetam (<25 %). Percent recovery at 3 different concentrations (10 ng/mL, 40 ng/mL, and 400 ng/mL) was >80 % for all analytes except Levetiracetam at all three concentrations, Oxcarbazepine at 40 ng/mL, and Topiramate at 10 ng/mL. Percent CVs were within ±20 % for all analytes (**Table 12**). A 100-fold recovery improvement was observed for Topiramate from the lowest concentration (10 ng/mL) to the highest concentration (400 ng/mL). However, this loss of recovery may be an artifact of poor MS signal at the lower concentrations.

Matrix Effect was 34 % to 68 % at 10 ng/mL. It improved to 80±5 % at 40 ng/mL, except Topiramate (95 %) and Carbamazepine Epoxide (112 %). At 400 ng/mL, Matrix Effect was 100±10 % except Carbamazepine Epoxide (251 %). Process Efficiency was 33 % to 59 % at 10 ng/mL for all analytes

except Carbamazepine Epoxide (85 %). Process Efficiency was 50±10 % at 40 ng/mL except Oxcarbazepine (62 %), Topiramate (86 %), and Carbamazepine Epoxide (164 %). At 400 ng/mL, Process Efficiency was 100±20 % except Oxcarbazepine (74 %) and Carbamazepine Epoxide (382 %).

Calibration curves over a 100-fold dynamic range (5 ng/mL to 500 ng/mL) with 1/x weighting demonstrated excellent linearity with R^2 values ≥0.993 for all target compounds including the Zwitterions Pregabalin and Gabapentin (**Figure 12**). The only exception Levetiracetam (R^2 = 0.976) with <25 % recovery (**Table 10**).

LC Conditions

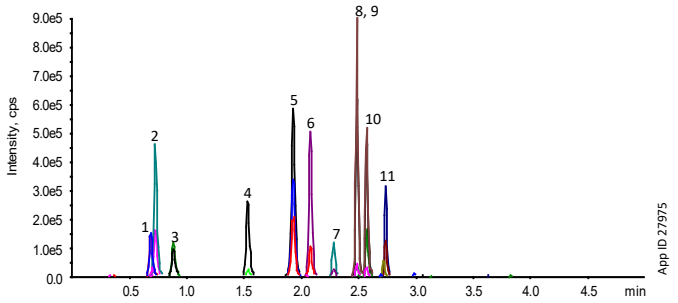
Column: Luna™ Omega 3 µm Polar C18
Dimensions: 50 x 3.0 mm
Part No.: 00B-4760-Y0
Mobile Phase: A: 2 mM Ammonium Acetate
B: 2 mM Ammonium Acetate in Methanol

Gradient:

Time (min)	% B
0	20
0.5	20
1.5	40
2.5	80
3	95
3.5	95
3.51	20
5	20

Flow Rate: 0.8 mL/min
Injection Volume: 5 µL
Temperature: 40 °C
LC System: Agilent® 1260 Infinity
Detection: MS/MS
Detector: SCIEX® 6500 Triple Quad™

Figure 11. Analysis of Anticonvulsants Extracted from Serum Using Strata™-X-CW Microelution Plate on a Luna Omega 3 µm Polar C18 Column. Spiked Concentration of Antipsychotics in Serum is 30 ng/mL.



Peak No.	Analyte	Retention Time (min)	Polarity	Peak No.	Analyte	Retention Time (min)	Polarity
1	Pregabalin	0.7	Positive	7	Lamotrigine	2.28	Positive
2	Gabapentin	0.8	Positive	8	Carbamazepine Epoxide	2.5	Positive
3	Levetiracetam	0.88	Positive	9	Topiramate	2.5	Negative
4	Zonisamide	1.53	Positive	10	Oxcarbazepine	2.6	Positive
5	Lacosamide	1.93	Positive	11	Carbamazepine	2.8	Positive
6	Felbamate	2.08	Positive				

Table 10. Recovery Comparison of Anticonvulsants Extracted from Serum Using the Strata-X-C SPE of the Strata-X Method Development 30 mg/well 96-Well Plate and the Strata-X-CW Microelution 2 mg/well 96-Well Plate.

	Strata-X-C SPE Sorbent of the Strata-X Method Development 30 mg/well 96-Well Plate *Spiked conc. of serum = 1 ng/mL		Strata-X-CW Microelution 2 mg/well 96-Well Plate *Spiked conc. of serum = 10 ng/mL	
	Elution Solvent: Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v)		Elution Solvent: 5 % Ammonium Hydroxide in Methanol	
Analyte	% Recovery	% CV (N = 4)	% Recovery	% CV (N = 4)
Pregabalin	53	9.0	95	17.3
Carbamazepine	82	3.0	88	12.5
Felbamate	31	17.6	91	11.2
Gabapentin	50	8.9	87	13.6
Lacosamide	31	21.6	80	13.8
Lamotrigine	76	7.6	98	17.3
Zonisamide	25	17.1	85	14.3
Oxcarbazepine	37	17.4	97	19.4
Carbamazepine Epoxide	96	2.9	124	15.8
Topiramate	26	54.1	54.9	20.1
Levetiracetam	<25	N/A	<25	N/A

Table 11. SPE Method Comparison of Anticonvulsants from Serum Using the Strata™-X-C SPE of the Strata-X Method Development 30 mg/well 96-Well Plate (Left) and the Strata-X-CW Microelution 2 mg/well 96-Well Plate (Right).

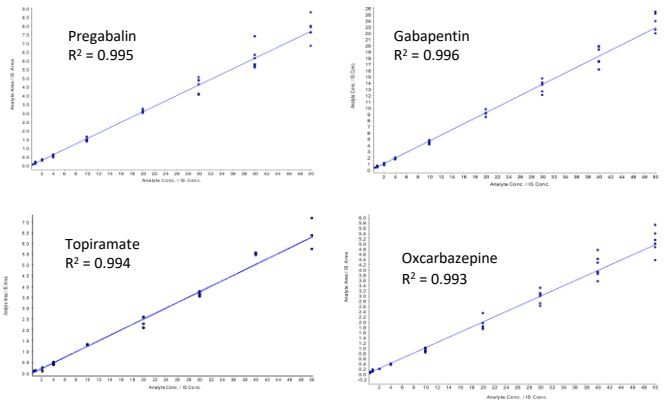
Step	Description
Sample Pretreatment:	500 µL human serum was spiked with Anticonvulsants standard mix at a concentration of 1 ng/mL then diluted with 1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted.
Condition:	Strata-X Method Development 96-well plate, 30 mg/well (Part No.: KS0-8209) with 1 mL of Methanol.
Equilibrate:	1 mL Water.
Load:	About 1.5 mL of pre-treated sample.
Wash 1:	1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted.
Wash 2:	1 mL Methanol / Water (1:1, v/v).
Dry:	5-8 minutes at 20-25 in. Hg.
Elute:	2 aliquots of 300 µL of Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v).
Dry Down:	15-20 minutes at 40 °C under a gentle stream of Nitrogen. Time ≅ 25 min Total Dry Time ≅ 35 min
Reconstitution:	500 µL on initial mobile phase spiked with 5 ng/mL Internal Standard mix (Carbazepine-D_{10}, Gabapentin-D_{10}, Topiramate-D_{12}). Time ≅ 1 min
Total Evaporation and Reconstitution Time:	≅ 36 min/plate
Total Reagent Volume:	6.1 mL/well ; 585.6 mL/plate

Step	Description
Sample Pretreatment:	10 µL human serum was spiked with Anticonvulsants standard mix and internal standards and then diluted with 200 µL of 25 mM Ammonium Formate, pH ~3.5 adjusted.
Condition:	Strata-X-CW Microelution 96-well plate, 2 mg/well (Part No.: 8M-S035-4GA) with 200 µL of Methanol.
Equilibrate:	200 µL Water.
Load:	200 µL diluted pre-treated sample.
Wash 1:	200 µL of Acidic Buffer 25 mM Ammonium Formate, pH ~3.5 adjusted
Wash 2:	200 µL Methanol / Water (2:8, v/v).
Dry:	1 minute at 20-25 in. Hg.
Elute:	2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Methanol.
Dry Down:	Bypass. Total Dry Time ≅ 1 min
Reconstitution:	Bypass.
Dilute:	Dilute with 200 µL mobile phase A (2 mM Ammonium Acetate) before injection.
Total Evaporation and Reconstitution Time:	≅ 1 min/plate
Total Solvent Volume:	1.3 mL/well ; 124.8 mL/well

Table 12. Method Performance and Qualification Data Utilizing Optimized Conditions and the Strata-X-CW Microelution Plate for the Extraction of Anticonvulsants from Serum.

Analyte	Concentration (ng/mL)	% Recovery	% CV	% Matrix Effect	PE	Precision	Accuracy	Linear regression R^2	Regression Equation
Pregabalin	10	95	17.3	52	49	12.8	102	0.995	y = 0.154 x + 0.0246
	40	98	3	84	50	3.1	101		
	400	105	5.1	98	100	10.8	101		
Carbamazepine	10	88	12.5	54	48	13.8	91	0.997	y = 0.162 x + 0.0656
	40	87	6.6	83	51	7.5	98		
	400	101	5.4	103	104	5.9	103		
Felbamate	10	91	11.2	56	51	5.4	85	0.996	y = 0.39 x + 0.14
	40	88.2	9.6	83	52	9.8	97		
	400	106.2	8.3	107	116	8.2	105		
Gabapentin	10	87	13.6	58	51	15.5	95	0.996	y = 0.454 x + 0.168
	40	94	4.5	85	47	5.8	99		
	400	98.7	8.5	102	98	8.6	101		
Lacosamide	10	80.2	13.8	57	45	5.9	86	0.995	y = 0.498 x + 0.173
	40	86.2	11.7	83	50	11.7	98		
	400	101.7	7	107	112	6.5	104		
Lamotrigine	10	98	17.3	60	59	14.1	95	0.994	y = 0.09 x + 0.040
	40	90	14.3	85	55	14.7	99		
	400	105	8.5	110	119	7.7	104		
Zonisamide	10	85	14.3	58	49	20	78	0.995	y = 0.236 x + 0.0888
	40	89	6.2	82	50	6.3	101		
	400	106	8.2	104	112	7.6	107		
Oxcarbazepine	10	96.5	19.4	34	33	4	85	0.993	y = 0.0987 x + 0.0404
	40	71	11.8	77	62	12	95		
	400	85	8.5	78	74	10.7	104		
Carbamazepine Epoxide	10	124.1	15.8	68	85	14.7	97	0.994	y = 0.502 x - 0.0569
	40	133	11.8	112	164	11.8	103		
	400	152	8.6	251	382	7.8	105		
Topiramate	10	54.9	51	68	37	78	70	0.994	y = 0.126 x + 0.0245
	40	92	31.7	95	86	36	109		
	400	109	22.2	109	106	20	105		
Levetiracetam	10	**<25**	-	-	-	-	-	0.9766	y = 0.124 x + 0.159
	40	**<25**	-	-	-	-	-		
	400	**<25**	-	-	-	-	-		

Figure 12. Calibration Curves for Four Selected Anticonvulsants Over 100-Fold Dynamic Range (5 ng/mL to 500 ng/mL).



Summary

This white paper outlines multiple sample preparation and LC-MS/MS conditions for the analysis of TDM drug analytes in serum for clinical research and forensic toxicology. Kinetex™ core-shell and Luna™ Omega columns, combined with SPE for sample clean up, can provide fast, sensitive, specific quantitation of drug analytes by LC-MS/MS for a large panel.

Need a different column size or sample preparation format?
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