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Validation of a High-throughput Screening and Quantification Method for the Determination of Gabapentinoids in Blood Using a Combination of LC-TOF-MS and LC-MS-MS

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Abstract

Gabapentinoids such as gabapentin (GP) and pregabalin (PGL) have been used to treat a wide range of neurological and psychiatric disorders. In recent years, there has been an increasing awareness of GP and PGL misuse among individuals with a history of polysubstance use. Both GP and PGL are understood to potentiate the effects of opioids, with fatalities involving GP and PGL being reported with increasing frequency. An efficient procedure was developed to screen and quantitate GP and PGL in blood samples using a combination of liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) and liquid chromatography tandem mass spectrometry (LC-MS-MS). The developed LC-MS-MS method was linear from 0.5–50 mg/L, with a limit of detection (LOD) of 0.1 mg/L for GP and PGL. An LOD of 0.5 mg/L was determined for both analytes on the LC-TOF-MS screen. A total of 1,091 blood specimens were subjected to a protein crash with methanol, in the presence of deuterated internal standards, PGL-d6 and GP-d10, to minimize the effects of varying matrix conditions. Specimens tested included both post-mortem blood and preserved blood specimens collected for the purposes of investigating drug-impaired driving and suspected drug-facilitated crimes. Of the total of specimens tested, 101 (9.3%) screened positive using the developed LC-MS-MS method for GP while only 13 (1.2%) blood specimens screened positive for PGL. All (100%) of the cases that screened positive for GP and PGL were confirmed positive by LC-MS-MS. Blood concentrations of GP and PGL ranged from <0.5 to 215 mg/L and from <0.5 to 32 mg/L, respectively. Of the blood specimens that had previously screened negative by LC-TOF-MS, 10% (N = 100) were randomly selected and tested by LC-MS-MS with 100% confirmed negative for GP and PGL. The developed methods provide a fast and reliable high-throughput screening and confirmation testing strategy for the detection of GP and PGL in blood specimens.

Introduction

Gabapentinoids are widely used in neurology, psychiatric and primary healthcare and are thought to possess GABA-mimetic properties that affect the dopaminergic reward system [1]. Gabapentin (GP) and pregabalin (PGL) are two such gabapentinoids currently being prescribed to treat various neurological and psychiatric disorders, but it has been noted that individuals with history of polydrug use can...
misuse GP and PGL [1]. In recent years, there has been increasing concern for the potential misuse of GP and PGL, which appears to be more prevalent among current or past opioid misusers [1,2]. A recent study showed that patients with opioid use disorder demonstrated higher GP and PGL abuse rates [1]. PGL has been misused due to its euphoric effects, rapid absorption and fast onset of action, which has been described by users to be similar to alcohol, benzodiazepines and gamma-hydroxybutyrate (GHB) but with enhanced euphoria [3]. Fatalities have occurred and are now increasingly common especially when combined with opioids drugs [1,2,3]. PGL has been detected with at least one other drug most commonly opioids and present in drug-dependent individuals [3]. At high doses, GP elicits similar effects to PGL, and a number of publications report higher rates of abuse of GP than PGL. [2,4–11]

Analytical testing, calibration ranges, limits of detection (LODs) and quantitation for GP and PGL varied among different laboratories [12,13,14]. It is important to note that the majority of laboratories screen and confirm GP with LC-MS/MS [12]. One of the main reasons is probably due to its increase sensitivity and selectivity. On the other hand, LC-TOF-MS coupled with electrospray ionization provides accurate mass identification of analytes, in addition to having the ability to data mine [15]. Data mining of known and unknown compounds at a later time has significant benefits as new investigative information comes to light [15–16]. Although, GP and PGL can be analyzed in different biological matrices, the main interest of this study was to analyze post-mortem (PM) blood specimens and blood samples collected from individuals suspected of being impaired when driving or from drug-facilitated crimes (DFC). A study conducted over a period of 2 years using PM blood samples reported concentrations ranging from 0.4 mg/L to 17 mg/L for PGL in prescribed individuals [3]. In a case report, the presence of GP due to acute intoxication was found at concentrations greater than 20 mg/L. GP therapeutic blood concentrations less than 10 mg/L have been reported for individuals prescribed a range of doses [17].

The aim of this study was to develop a simple method for screening and confirming the presence of GP and PGL in blood specimens across a wide range of concentrations to encompass the therapeutic and toxic ranges. Within the New York City Office of Chief Medical Examiners Forensic Toxicology Laboratory, GP/PGL analysis was previously scheduled as a targeted test in cases where there was a suspicion of misuse or to confirm compliance with prescribed medication. Due to the increased awareness of GP and PGL misuse, both drugs are now included as part of the comprehensive screening panel so we can better understand the extent of misuse of these drugs. The described method was adapted from a previously published procedure used for the analysis of antiepileptic drugs in PM blood sample and utilizes only 100 μL of blood [13]. The method was validated to screen and quantify GP and PGL using LC-TOF-MS and LC-MS/MS in accordance with industry standards.

Materials and Methods

Chemicals

GP, PGL (1 mg/mL), GP-D<sub>10</sub> and PGL-D<sub>6</sub> (100 μg/mL) were purchased from Cerilliant Corporation (Round Rock, TX, USA). Acetonitrile (LC-MS grade), deionized/HPLC grade water and methanol (LC-MS grade) were purchased from Spectrum Chemical (New Brunswick, NJ, USA). Formic acid was purchased from TCI America (Portland, OR, USA).

Instrumentation and chromatographic conditions

LC-TOF-MS

Samples were analyzed using an Agilent LC-TOF-MS series 6230 coupled with a 1290 HPLC. Mobile phases of water and acetonitrile, both with additions of 0.1% formic acid, were used and run in a gradient (Table I) on a Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 μm), with a column temperature of 45°C and a constant flow rate of 0.3 mL/min. Autosampler injection volume was set at 2.5 μL, with an automated needle wash of 3 s prior to each injection. Sample run time was 8.00 min with a 5-min post-run time. The Agilent Jet-Stream Dual ESI ion source was operated in positive polarity, with a TOF acquisition mass range of 100–1700 m/z. MassHunter software (B.06.00) was utilized to acquire data on this instrument. MassHunter Qualitative Analysis (B.07.00) was used to process all data, using the Find By Formula search algorithm, with a customized database created with Agilent PCDL Manager. Table I contains a detailed list of parameters for this LC-TOF-MS method.

LC-MS-MS

Quantitation of GP and PGL was accomplished on a 1200 Infinity Series liquid chromatography system coupled to a 6460 Triple Quad Mass Spectrometer manufactured by Agilent Technologies (Santa Clara, CA). Separation was achieved using an Agilent Infinity Lab Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 μm) coupled with a guard column that was held at 55°C with a flow rate of 0.7 mL/min. Mobile phase A & B previously described under LC-TOF-MS was also utilized for the LC-MS-MS. A total of 5 μL of sample was injected and monitored using dynamic multiple reaction monitoring (dMRM) mode for 6.10 min with a 2-min post-run time, for a total run time of 8.10 min. The starting conditions were 95% MPA: 5% MBP. At 4 min MBP increased to 8% B and then ramped to 100% B at 4.10 min where it was held for 2 min. Precursor ions, product ions, fragmentor voltage and collisionenergies for GP and PGL and their respective deuterated internal standards were optimized using MassHunter Optimizer software. Masshunter Data Acquisition software (version B.08.00) was used for acquisition data collection and instrument control, and QQQ Quantitative Analysis software (version B.07.01) was used to analyze data. Table II contains a detailed list of parameters for this LC-MS-MS method.

Preparation of calibrators and controls

Two working calibrator solutions containing GP and PGL were prepared at 20 mg/L and 100 mg/L in methanol and two control working solutions at 20 mg/L and 100 mg/L. The internal standard pool of PGL-d<sub>4</sub> and GP-d<sub>10</sub> was prepared at 2.5 mg/L in methanol. Working solutions were stored refrigerated at 2–8°C when not in use. A total of 100 μL sample size of blood was used for both methods.

A single-point calibrator was spiked at 5 mg/L (see Figure 1) for the LC-TOF-MS screening method, using a 20-mg/L working solution, and two positive controls were spiked at 0.5 mg/L and 30 mg/L, using 20 mg/L and 100 mg/L working solutions, respectively. A drug-free (negative) control was included in each batch.

For the LC-MS-MS method, seven calibrators at concentrations of 0.5 mg/L, 1 mg/L, 2 mg/L, 5 mg/L, 10 mg/L, 25 mg/L and 50 mg/L were spiked into drug-free blood and aliquoted into 1.5-mL polypropylene microcentrifuge tubes. The 20-mg/L calibrator solution was used to spike the four lowest calibrators, and the 100 mg/L was used to spike the final three calibrators. Quality controls were spiked at low, mid and high concentrations (3 mg/L,
Table I. LC-TOF-MS acquisition parameters

<table>
<thead>
<tr>
<th>Source:</th>
<th>Source Parameters:</th>
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<tr>
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<tr>
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<td>Binary pump (LC):</td>
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<td>Flow:</td>
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<td>6</td>
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<tr>
<td>Post time:</td>
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Table II. LC-MS/MS acquisition parameters

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<td>8</td>
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<tr>
<td>Post time:</td>
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<tr>
<td>5</td>
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</tbody>
</table>

15 mg/L and 30 mg/L, respectively) using the working solution at 100 mg/L. A drug-free (negative) control was aliquoted using 100 μL of drug-free calf blood.

Sample preparation
A total of 100 μL of blood was aliquoted into microcentrifuge tubes. To all samples (including calibrators and QCs) 100 μL of 2.5 mg/L PGL-d6 and GP-d10 internal standard solution was added, then immediately capped and vortexed for 3 s. A total of 300 μL of methanol was added to each tube and samples, vortexed thoroughly for 30 s, then centrifuged at 10,000 rpm for 10 min.

Method validation
The method was validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation [16] and the following parameters were assessed:
Validation of Gabapentinoids in Blood Using a Combination of LC-TOF-MS and LC-MS-MS

Figure 1. LC-TOF-MS chromatogram of pregabalin and gabapentin at 5 mg/L.

Linearity, LOD and quantitation, bias and precision, interference studies, ion suppression/enhancement, carryover, dilution integrity and stability. Case sample comparison studies between the LC-TOF-MS and the LC-MS-MS were also included in this validation using 1,091 blood specimens (982 PM, 108 driving while impaired and 1 DFC).

**Linearity**
Seven calibrators spanning the range of 0.5–50 mg/L were run for 5 days on the LC-MS-MS, and the best-fit model for each analyte was determined mathematically using the results of residual value deviations. Residual deviations were observed visually on a graph with all 5 days of data plotted together for linear vs. quadratic models. Different weighting models were explored for each analyte for the best weighted fit. Once the best fit was determined the coefficient of variation was calculated.

**LODs and quantitation**
For the LC-TOF-MS an administratively defined LOD value was established (0.5 mg/L); this concentration was run in triplicate over 5 batches, and a total of 15 samples were run. For the LC-MS-MS, the LOQ was defined as the lowest calibrator concentration, 0.5 mg/L; the LOD was tested at 0.1 mg/L. In order for the LOD to pass, the instrument’s response must be >3× the noise level in the drug-free negative control.

**Bias and precision**
Analysis of variance (ANOVA): single factor analysis was performed to calculate the bias and precision of three QC concentrations: low QC 3 mg/L, mid QC 15 mg/L and high QC 30 mg/L. These QCs, spanning the entire analytical range 0.5–50 mg/L on the LC-MS-MS, were monitored in triplicate over five runs. The within-run and between-run of a total of 15 replicates were also calculated.

**Interference studies**
A total of 10 different sources (9 PM bloods & 1 calf blood) without the addition of an internal standard were analyzed for matrix interferences on the LC-TOF-MS. Furthermore, commonly found analytes to the laboratory that include drugs of abuse (DOA) were evaluated by fortifying drug-free blood with drug reference materials at various concentrations. Stable-isotope and analyte interference studies were also performed.

For the LC-MS-MS, the presence of interferences was evaluated using a low QC sample fortified with high concentrations of commonly found DOA such as benzodiazepines, synthetic opioids, amphetamines, synthetic cathinones, cocaine, opiates, methadone and cannabinoids.

**Ion suppression/enhancement**
During the sample preparation, 14 drug-free blood samples (12 PM, 2 commercially sourced) were fortified after the addition of methanol with analytes at a low concentration, 0.5 mg/L, and at a high concentration, 40 mg/L, in order to determine the amount of ionization suppression or enhancement present using the LC-TOF-MS.

For the LC-MS-MS, the presence of interferences was evaluated using a low QC sample fortified with high concentrations of commonly found DOA such as benzodiazepines, synthetic opioids, amphetamines, synthetic cathinones, cocaine, opiates, methadone and cannabinoids.

**Carryover**
A drug-free control was injected immediately after the upper limit of quantitation (ULOQ) of 50 mg/L and also at 2xULOQ (100 mg/L) and evaluated for carryover. Both sets of concentrations and drug-free (negative) control were performed in triplicate.

**LC-MS-MS dilution integrity**
Dilution integrity was explored by performing a 1:2 and 1:5 dilution of an 80 mg/L spiked sample in drug-free negative matrix. The integrity was tested by diluting the 80 ng/mL original sample in triplicate into drug-free negative blood as well as into deionized water.
Figure 2. LC-MS/MS chromatogram of PGL at 0.52 mg/L and GP at 0.1 mg/L.

### Table III. Bias and precision of gabapentin and pregabalin in blood

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Low QC %CV</th>
<th>Mid QC %CV</th>
<th>High QC %CV</th>
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<tbody>
<tr>
<td></td>
<td>Within-run</td>
<td>Between-run</td>
<td>Within-run</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>2.88</td>
<td>3.38</td>
<td>2.76</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>3.18</td>
<td>3.48</td>
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### Table IV. Matrix effect, process efficiency and extraction efficiency for gabapentin and pregabalin

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<tr>
<th></th>
<th>Gabapentin</th>
<th>Pregabalin</th>
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<tr>
<td>Ion Suppression/Enhancement</td>
<td>13.7</td>
<td>14.9</td>
</tr>
<tr>
<td>%RSD</td>
<td>7.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Extraction Efficiency</td>
<td>38.9</td>
<td>39.36</td>
</tr>
<tr>
<td>Process Efficiency</td>
<td>44.22</td>
<td>45.24</td>
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</table>

### Stability

The stability of extracted samples was evaluated by re-injecting the low, mid and high QCs after 24 h and 48 h on the auto-sampler, maintained at room temperature.

### Method comparison

**LC-TOF-MS vs LC-MS-MS**

Toxicological analysis of GP and PGL was requested on PM, road traffic (RT) and sexual assault (SA) blood cases as part of an initial comprehensive testing panel offered by the laboratory during a 4-month period from 1 October 2018 to 31 January 2019. A total of 1091 RT, SA and PM blood sample cases were initially screened for GP and PGL using LC-TOF-MS. All samples that screened presumptive positive for either analyte were scheduled for confirmation by LC-MS-MS. In addition, 100 blood specimens that screened negative by LC-TOF-MS for GP and PGL were randomly chosen for reanalysis by LC-MS-MS.

### Results and Discussion

#### Linearity

The linear ranges for GP and PGL were determined to be 0.5–50 mg/L. Both analytes had a correlation coefficient of \( r^2 > 0.999 \) with a %CV < 20%, and the weighting was demonstrated to be 1/X for GP and 1/x^2 for PGL.

#### Limits of detection and quantitation

GP was detected in all 15 samples for the administratively defined LOD concentration (0.5 mg/L) using the LC-TOF-MS, while only 14 samples were detected for PGL. According to the SWGTOX guidelines when making an administratively defined decision point, a minimum of three samples per run over three runs are sufficient to demonstrate the detection and identification criteria of analytes [18]. PGL was detected in 14 out of 15 samples when determining the LOD. All LOD results for PGL were within 5 ppm, and 11 of 14 LOD results for GP were within 5 ppm. To meet criteria for a “presumptive positive,” the analyte retention time had to be ±5%.
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Figure 3. Distribution of gabapentin concentrations in PM blood (All sources vs femoral only).

from the target, with a mass error of <10 ppm and acceptable peak shape for the analyte(s).

For the LC-MS-MS, the LOQ for GP and PGL was determined to be 0.5 mg/L. Acceptable criteria were met for concentration, responses, quantifier and qualifier ions, as illustrated in Figure 2 for a PM blood specimen. The LOD at 0.1 mg/L had acceptable responses, retention time, ion ratio and peak shape. Accuracy and precision for GP had a within-run %CV of 2.55 and between-run %CV of 6.21. PGL had a within-run %CV of 3.75 and the between-run %CV of 6.21.

LC-MS-MS bias and precision
Using ANOVA single factor analysis, the developed method showed that both bias and precision did not exceed 20% for either analytes. See Table III below for the %CV for low, middle and high concentration QC.

Interference studies
In 10 different blood sources tested, no matrix interferences were observed for the LC-TOF-MS. There was no stable isotopic contribution, and no interferences were observed from common analytes tested.

No exogenous interference was observed on the LC-MS-MS when the low QC was spiked with common DOA. No interferences from common DOA were found from high levels of methadone at 3000 ng/mL, heroin and cocaine at 1500 ng/mL, sympathomimetic amines and synthetic cathinones at 1500 ng/mL, synthetic opioids at 1500 ng/mL, benzodiazepines at 500 ng/mL and THC at 100 ng/mL. The low QC accuracy was within 20% for both analytes, and the ion ratios were within range as well. The low QC was still in range with accuracy values of 80–120% and transition ratios passed for both analytes.

Ion suppression/enhancement
For the low concentration, 0.5 mg/L, an enhancement of 171% and 176% was observed for PGL and GP, respectively, on the LC-TOF-MS. At the high concentration, 40 mg/L, an enhancement of 112% and 108% was observed for PGL and GP, respectively.

On the LC-MS-MS, ion suppression/enhancement (matrix effect) was tested concurrently with process efficiency and extraction efficiency. The target goal for this parameter is the mean matrix effect not to exceed 25%. There was no significant matrix effect exhibited. The results are summarized in Table IV.

Carryover
The drug-free negative control injected, at three different times, after the high concentrations 100 mg/L were evaluated and concluded to be free from the presence of either GP or PGL.

LC-MS-MS dilution integrity
Drug-free negative blood control and deionized water were both used to explore a 1:2 and 1:5 dilution of a prepared 80 mg/L spiked sample. Comparing the concentration of GP and PGL over three repeats showed %CV below 10% when the sample was diluted into blank blood matrix. Water dilutions did not show accurate concentration when compared to the results where drug free- blood was used. It was determined that drug-free blood should be used for dilutions due to the consistency of compound results in this matrix. GP PM samples that exceeded the ULOQ and when more than one dilution was needed on the same sample, the GP results where drug-free blood was used showed accurate and precise results.

Stability
The stability of the extracted QC samples at low, mid and high concentration (3 mg/L, 15 mg/L and 30 mg/L, respectively) were analyzed at 24 h and 48 h on the autosampler that is kept at room temperature. PGL at all three concentrations at 24 h and 48 h had an area response within 3% difference of the fresh QC responses. GP also calculated within 3% difference for all concentrations at 24 h and 48 h, except for 15 mg/L at 48 h in the autosampler that was ~8.79% difference compared to the fresh QC.

Method comparison
GP and PGL were identified as presumptive positive in 101 and 13 blood specimens, respectively, using the developed LC-TOF-MS method and were all confirmed positive using the developed LC-MS-MS method. An additional 100 randomly chosen blood specimens that initially screened negative by LC-TOF-MS were reanalyzed by LC-MS-MS, and all were confirmed negative. The concentrations measured ranged from <0.5 mg/L to 215 mg/L for GP and <0.5 mg/L to 32 mg/L for PGL.

The majority of blood specimens confirmed positive for GP or PGL were from PM blood specimens with femoral blood the most
common source at 78% (N = 87). Other blood sources included heart, cavity, jugular, subclavian and decomposition fluid.

There were only three cases positive for GP or PGL from drivers suspected of driving while impaired (0.93 mg/L and 4.1 mg/L GP; 4.2 mg/L PGL) and one DFC case (<0.5 mg/L PGL).

Figure 3 below illustrates the distribution of GP concentrations in PM blood comparing all blood sources (N = 95) to femoral blood only (N = 80). A detailed evaluation of the significance of these concentrations in relation to the case is beyond the scope of this study and requires additional demographic information including the circumstances leading to death, the prescription history and other drugs detected. However, what the data does provide is the ability to assess the use and potential misuse of both GP and PGL in deaths occurring within the City of New York. There is also a significant difference in prevalence between the drugs with GP present in approximately eight times as many cases as PGL and 40% of GP concentrations in excess of 10 mg/L. This may reflect the prevalence of GP prescriptions compared with those for PGL or that GP is being misused to a greater extent than PGL. The latter may be a more likely explanation as drug seizures in the New York/New Jersey region frequently report GP but not PGL.

Conclusion

The New York City Office of Chief Medical Examiners Forensic Toxicology Laboratory is one of the largest forensic toxicology laboratories in the US with over 6000 cases per year. The described method combines the use of LC-TOF-MS as an initial screening test to eliminate negative cases, followed by confirmation of positives by LC-MS-MS. The validated methods have proved to be effective for detection of GP and PGL in blood specimens utilizing a testing strategy that reduces the already high volume of tests scheduled for LC-MS-MS analysis. With 100% correlation between the LC-TOF-MS and LC-MS-MS instruments for both positive and negative cases achieved, this provides laboratories with an alternative testing option to targeted testing or screening and confirmation by LC-MS-MS.

References