# Analysis of Prilocaine in Postmortem Whole Blood and Vitreous Fluid Utilizing UPLC®/TOFMS (Screening) and UPLC®/MS/MS (Confirmation)

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## Abstract

A case study is presented that involves the death of a ten year-old girl following a visit to the dentist's office for a routine oral surgery. The drug administered for anesthesia was Prilocaine, which is a local anesthetic that is closely related to the more common drugs Lidocaine, Bupivacaine, and Mepivacaine. It is available in an injectable form (trade name Citanest®). While in the dentist's chair, the subject became unresponsive after administration of a premeasured dosage of Prilocaine, along with nitrous oxide. The types of specimens that were analyzed in this case included whole blood and vitreous fluid collected at the autopsy.

First, a novel method was developed for unknown forensic toxicological analysis utilizing a Waters Acquity UltraPerformance Liquid Chromatograph (UPLC®) coupled to a Waters LCT Premier XE time of flight mass spectrometer (TOFMS). The specimens were extracted by a deproteinization with acetonitrile, which contained an internal standard. A gradient elution of 0.05% formic acid in DI water and methanol was performed on a Waters Acquity HSS T3, 2.1 x 100 mm, 1.7 µm particle size analytical column. Total run time for one specimen was 6.5 minutes. Prilocaine was identified using exact mass and retention time as library filters.

Second, a novel confirmatory method was developed for the analysis of Prilocaine utilizing a Waters Acquity UPLC® coupled to a Waters Tandem Quadrupole Detector (TQD) mass spectrometer. Similar to the general screening method, the extraction of the analyte of interest was carried out by the deproteinization of the whole blood specimens with acetonitrile, which contained a deuterated analog of Lidocaine as an internal standard. A full method validation was completed. The linear range of the analysis was 0.5 µg/mL-20 µg/mL. Quality control specimens were spiked at 1.5 µg/mL, 4 µg/mL, and 16 µg/mL. A gradient elution of 0.1% formic acid in acetonitrile was performed on a Waters Acquity HSS T3, 2.1 x 150 mm, 1.7 µm particle size analytical column. Total run time for one specimen was 6 minutes.

## Introduction

Prilocaine, an amino amide, was first synthesized in 1960 as a local anesthetic. The trade name for Prilocaine is Citanest®. Prilocaine is also combined with equal amounts of lidocaine to form a topical anesthetic mixture that is available in cream or dermal patch forms. Although the metabolic pathway of Prilocaine in humans has not been investigated, in rodent models, the drug is quickly metabolized by hydrolysis of the amide linkage, which creates the metabolite o-toluidine. One hour after an injection is administered, the highest concentrations of Prilocaine are found in decreasing order in the following: lung, kidney, brain, heart, liver, and blood. Only a small amount of Prilocaine is excreted unchanged in the urine. Overdosage with Prilocaine can cause: dizziness, blurred vision, tremors, drowsiness, hypotension, bradycardia, convulsions, coma, and respiratory and cardiac arrest. Methemoglobinemia occurs in all patients who are administered the drug due to the metabolization of Prilocaine into o-toluidine. One case reported involves an 83 year old male who died after receiving 864 mg of Prilocaine during the course of an hour for dental anesthesia. The postmortem concentration of Prilocaine was 13 mg/L in blood (Baselt 929).

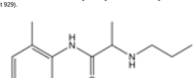

Figure 1: Structure of Prilocaine (Wikipedia)

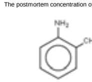


Figure 2: O-Toluidine (Wikipedia)

A pre-existing and validated screening method was utilized to screen the specimen by means of UltraPerformance Liquid Chromatography (UPLC®) coupled with Time of Flight Mass Spectrometry (TOFMS). UPLC® utilizes new technology of smaller column particle sizes, 1.7 or 1.8 µm. Traditional LC columns have particle sizes of 3.5 µm and 5 µm. The UPLC® also has integrated high-pressure fluidic modules. These upgrades over the traditional HPLC system yield a higher resolution and a much faster, more sensitive, and more robust chromatographic system for use in a toxicology laboratory. Time of flight mass spectrometry offers unsurpassed sensitivity and specificity, with accurate mass detection to four decimal places. In this type of mass spectrometer, all ions are accelerated through the same electrical field; ions of different masses will have different velocities. The time of flight of the ions is measured over a fixed distance to a detector. Ions of lighter masses travel faster and reach the detector in a shorter period of time while ions of heavier masses travel slower and reach the detector in a longer period of time. Utilizing this form of instrumentation for screening purposes allows the specimen to be screened for many therapeutic drugs, drugs of abuse, and other chemicals during a quick six minute run time.

A novel, confirmatory method was developed and validated specifically for the analysis of this case. UltraPerformance Liquid Chromatography (UPLC®) coupled with Tandem Mass Spectrometry (MS/MS) utilizes the same technology involving columns of smaller particle sizes joined with the specificity of a triple quadrupole detector mass spectrometer. The detector is two mass spectrometers separated by a collision cell. The first quadrupole (Q1) acts as a mass filter for ions of a specific mass to charge ratio. These ions are filtered out and passed onto a collision cell (Q2), where they collide with an inert gas (usually argon) and create smaller fragment ions. The second quadrupole (Q3) also acts as a mass filter and allows ions of specific mass to charge ratios to pass to the detector. The robustness and resolution of the UPLC® system coupled with the specificity and accuracy of the tandem mass spectrometer create a practical instrument for the confirmatory analysis of presumptive positive results for drugs of abuse, therapeutic drugs, and other chemicals in forensic toxicological analysis.

The objective of this project was to develop a new confirmatory method for specimens screening positive for Prilocaine utilizing the combinatorial approach of UPLC® and tandem mass spectrometry.

## Case History and Background

A case study is presented that involved the death of a ten year old female during a visit to the dentist's office for a routine oral surgery. The drug administered for anesthesia was Prilocaine, which is a local anesthetic that is closely related to the more common drugs Lidocaine, Bupivacaine, and Mepivacaine. It is available in an injectable form (trade name Citanest®), which is most often used in dentistry. While in the dentist's chair, the subject became unresponsive after administration of a premeasured dosage of Prilocaine, along with nitrous oxide. Whole blood and vitreous fluid were collected during the autopsy and sent to the laboratory for toxicological analysis.

## Methods of Analysis – UPLC®/TOFMS

### UPLC®

A Waters Acquity UPLC® was coupled to a Waters LCT Premier™ XE. The analytical column was an Acquity UPLC® HSS T3, 2.1 x 100 mm, 1.7 µm particle size, held at 30 ºC. A gradient elution of 0.05% Formic Acid in DI Water and Methanol (Optima grade) was performed. Specimens were held at 7.5ºC. Total run time for one specimen was 6.5 minutes.

| Time (Minutes) | Flow Rate (mL/minute) | Mobile Phase A (%) | Mobile Phase B (%) | Curve |
|---|---|---|---|---|
| 0.00 | 0.300 | 90.0 | 10.0 | - |
| 0.50 | 0.300 | 90.0 | 10.0 | 6 |
| 4.10 | 0.300 | 5.0 | 95.0 | 6 |
| 5.00 | 0.300 | 5.0 | 95.0 | 6 |
| 5.10 | 0.300 | 90.0 | 10.0 | 6 |
| 6.50 | 0.300 | 90.0 | 10.0 | 6 |

Table 1: The Acquity UPLC® Gradient

### Mass Spectrometer

The mass spectrometer (Waters LCT Premier™ XE) was operated in W-optics mode for higher resolution (>10,000 FWHM). In order to detect acidic, basic, and neutral analytes, scans were completed in both positive and negative electrospray ionization modes over a mass range of 50 Daltons to 1,100 Daltons. A low voltage scan (Aperture voltage = 10) was used for parent mass identification. Dynamic range enhancement was used to increase the linear range by an order of magnitude. Real time accurate mass data was acquired by reference to an independently sampled reference material (Leucine Enkephalin, [M+H]+ = 556.2771 Daltons.)

### Software

The software used for this assay was MassLynx, version 4.1 in combination with the QuanLynx application manager. QuanLynx is designed for quantitative analysis, but a method was developed for qualitative and semi-quantitative analysis, which contained each analyte and its accurate mass and retention time, along with integration and peak detection parameters.

### Extraction Procedure

A 250 µL aliquot of specimen was precipitated with Acetonitrile containing an internal standard (Proadifen, 10 ng/mL). The specimen was vortexed and centrifuged. The supernatant was transferred to a glass autosampler vial and injected on the instrument.

## Results – Screening by UPLC®/TOFMS

Time of flight mass spectrometry offers very good sensitivity and accurate mass calculation to four decimal places. Figure 3 shows the total ion chromatogram (TIC) of a negative blood specimen spiked with Prilocaine at 1 µg/mL. The theoretical accurate mass ([M+H]+) for Prilocaine is 221.1654 Daltons. The extracted ion chromatogram (XIC) details the mass to charge ratio extracted out of the TIC. A peak is detected at a retention time equal to 3.08 minutes. Figure 5 details the extracted ion spectrum (XIS) of the extracted peak. The calculated accurate mass of Prilocaine in the spiked blood specimen is 221.1686 Daltons. An XIC of a negative quality control specimen is shown in Figure 6 and shows that no discernable signal is detected.

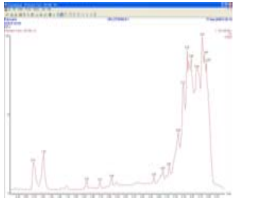

Figure 3: TIC of a specimen spiked with Prilocaine (1 µg/mL)

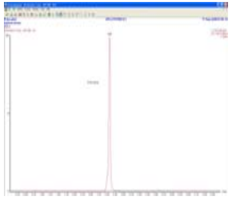

Figure 4: XIC of a blood specimen spiked with Prilocaine (1 µg/mL)

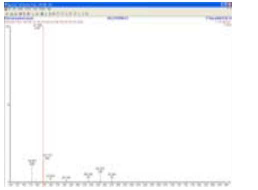

Figure 5: XIS of the XIC of the blood specimen spiked with Prilocaine (1 µg/mL)

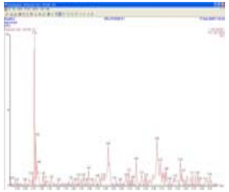

Figure 6: XIC of Prilocaine a negative quality control specimen.

The following figures detail the XIC and the XIS of the postmortem blood specimen analyzed in this case. The XIC of Prilocaine mass to charge ratio is detailed in Figure 7. The peak detected is at the exact retention time of the peak of interest in the spiked specimen. Figure 8 details the extracted ion spectrum (XIS) of the extracted peak of interest. The calculated accurate mass of Prilocaine in the postmortem blood specimen is 221.1679 Daltons.

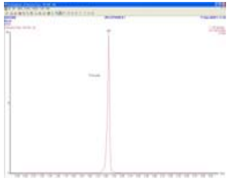

Figure 7: XIC of the postmortem blood specimen

Figure 8: XIS of the XIC of Prilocaine in the postmortem blood specimen.

The postmortem specimen can be considered presumptively positive for the presence of Prilocaine by retention time and accurate mass, within a certain mass accuracy tolerance. In order to confirm the presence and quantify this drug, further investigation is necessary by a more specific method.

## Methods of Analysis – UPLC®/MS/MS

### UPLC®

A Waters Acquity UPLC® was coupled to a Waters Tandem Quadrupole Detector (TQD). The analytical column was an Acquity UPLC® HSS T3, 2.1 x 150 mm, 1.7 µm particle size, held at 35 ºC. A gradient elution of 0.1% formic acid in DI water and 0.1% formic acid in acetonitrile was performed. Specimens were held at 7.5ºC. 2 µL of specimen was injected onto the instrument. Total run time for one specimen was 6 minutes.

| Time (Minutes) | Flow Rate (mL/minute) | Mobile Phase A (%) | Mobile Phase B (%) | Curve |
|---|---|---|---|---|
| 0.00 | 0.500 | 95.0 | 5.0 | - |
| 1.00 | 0.500 | 95.0 | 5.0 | 6 |
| 3.50 | 0.500 | 0.0 | 100.0 | 6 |
| 4.50 | 0.500 | 0.0 | 100.0 | 6 |
| 4.51 | 0.500 | 95.0 | 5.0 | 6 |

Table 2: The Acquity UPLC® Gradient

### Mass Spectrometer

The mass spectrometer (Waters TQD) was operated in positive electrospray ionization mode. Capillary voltage was 1.0 V. The primary ion transition for Prilocaine was 221.35 → 86.02 Daltons, with a cone voltage of 11 V and a collision energy of 14 eV. The secondary ion transition for Prilocaine was 221.35 → 136.02 Daltons, with a cone voltage of 11 V and a collision energy of 20 eV. The ion transition used for Lidocaine d10 was 245.10 → 96.2 Daltons, with a cone voltage of 25 V and a collision energy of 24 eV. Dwell times were 0.005 seconds.

### Software

The software used for this assay was MassLynx, version 4.1 in combination with the QuanLynx application manager, which allows for the monitoring of two ion transitions per analyte.

### Extraction Procedure

A 50 µL aliquot of specimen was precipitated with 4 mL of acetonitrile containing an internal standard (Lidocaine d10, 10 ng/mL). The specimen was vortexed and centrifuged. The supernatant was transferred to a glass autosampler vial and injected on the instrument.

## Validation

A full method validation was completed. Linearity, carryover, accuracy, precision, freeze/thaw stability, specimen extracts stability, and selectivity were all addressed.

### Linearity

The linear range of the assay was 0.5 µg/mL – 20 µg/mL. No true limit of detection (peak of interest with a signal to noise ratio of 3:1) was evaluated. It was noted during validation that the primary ion transition had an intensity of approximately 100 times the second ion transition. Calibration curve points were 0.5, 1, 2, 5, 10, and 20 µg/mL.

### Carryover

No carryover of the peak of interest was detected in a blank DI water specimen injected directly after a blood specimen spiked with 80 µg/mL of Prilocaine.

### Accuracy and Precision

Accuracy and precision were evaluated by analyzing five replicate specimens of three different values (1.5 µg/mL, 4 µg/mL, and 16 µg/mL) over the course of 4 days. All accuracy and precision data (Table 3) was excellent.

| Description | 1.5 µg/mL | 4 µg/mL | 16 µg/mL |
|---|---|---|---|
| Mean | 1.49 | 4.02 | 16.29 |
| Standard Deviation | 0.0865 | 0.1654 | 0.4555 |
| CV (%) | 5.79 | 4.11 | 2.80 |
| Accuracy (%) | 99.57 | 100.59 | 101.83 |
| RE % | 0.43 | -0.59 | -1.83 |

Table 3. Accuracy and Precision

### Freeze/Thaw Stability

In order to evaluate the effects of the freeze and thaw process on the analyte of interest, a freeze/thaw stability study was completed. Prilocaine was shown to be stable (Table 4.) through at least 3 freeze and thaw cycles.

| Description | 1.5 µg/mL | 4 µg/mL | 16 µg/mL |
|---|---|---|---|
| Mean | 1.43 | 3.98 | 15.36 |
| Standard Deviation | 0.0440 | 0.1246 | 0.2172 |
| CV (%) | 3.08 | 3.13 | 1.41 |
| Accuracy (%) | 95.26 | 99.61 | 96.01 |
| RE % | 4.74 | 0.39 | 3.99 |

Table 4. Freeze and Thaw Accuracy and Precision

### Specimen Extracts Stability

The specimen extracts were analyzed at times of 0 hours (initial), 8 hours, 24 hours, 48 hours, and 72 hours. Specimen extracts were proven to be stable in the autosampler over the 72 hour duration of time. No significant degradation was noted.

### Selectivity

Selectivity of the method was evaluated by analyzing specimens spiked with a total of 100 different analytes. No interferences were detected.

The following list details the analytes:

Alprazolam, Diazepam, Nordiazepam, Lorazepam, Oxazepam, Temazepam, Flunitrazepam, Chlordiazepoxide, Midazolam, Clonazepam, Amitriptyline, Nortriptyline, Doxepin, Nordoxepin, Imipramine, Desipramine, Fluoxetine, Norfluoxetine, Cyclobenzaprine, Clomipramine, Norclomipramine, Bupropion, Citalopram, Paroxetine, Sertraline, Norsertraline, Venlafaxine, Norvenlafaxine, Mirtazapine, Duloxetine, Quetiapine, Olanzapine, Clozapine, Norclozapine, Haloperidol, Chlorpromazine, Risperidone, Methadone, EDDP, Propoxyphene, Norpropoxyphene, Salicylic Acid, Ibuprofen, Naproxen, Acetaminophen, Amphetamine, Methamphetamine, MDMA, PMA, Pseudoephedrine, Ephedrine, Cocaine, Benzoylecgonine, Cocaethylene, Phenobarbital, Butalbital, Butabarbital, Secobarbital, Amobarbital, Pentobarbital, Mephobarbital, Caffeine, Cotinine, Nicotine, Methylphenidate, Dextromethorphan, Diphenhydramine, Chlorpheniramine, Doxylamine, Fentanyl, Tramadol, Nortramadol, Gabapentin, Pregabalin, Levetiracetam, Lamotrigene, Zonisamide, Phenytoin, Carbamazepine, Carbamazepine-10,11-epoxide, 10-monohydroxyoxcarbazepine, Primidone, Felbamate, Topiramate, Valproic Acid, Bupivacaine, Lidocaine, Carisoprodol, Meprobamate, Ketamine, Strychnine, Zolpidem, PCP, Alfentanil, Meperidine, Normeperidine, Thiopental, Verapamil, and Propranolol

## Results – Confirmation by UPLC®/MS/MS

Tandem mass spectrometry offers very good sensitivity and specificity. Figure 9 shows the chromatograms of the analyte of interest and internal standard. Figure 10 details a negative quality control specimen. No signal for Prilocaine is detected for Prilocaine's primary ion transition. Figure 11 details the chromatographic view of the postmortem specimen.

Figure 9: Chromatograms of an extracted Prilocaine spiked calibrator

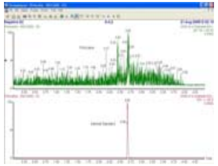

Figure 10: Chromatograms of an extracted negative quality control specimen.

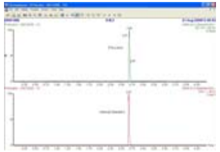

Figure 11: Chromatograms of the postmortem blood specimen

Figure 12: Onscreen view of the QuanLynx application manager

Figure 12 depicts the onscreen view of the QuanLynx application manager. The chromatograms of each of the ion transitions are shown in the lower left-hand corner of the screen, while the lower right hand corner houses the calibration information. The upper portion of the screen details all specimen information, including names, retention times, responses, deviation percentages, flags, and calculated concentrations. The postmortem whole blood specimen was positive for Prilocaine at a concentration of 1.8 µg/mL. The postmortem vitreous fluid specimen was positive for Prilocaine with a concentration of 0.7 µg/mL.

## Application and Conclusion

A novel, rapid UPLC®/TOFMS method was used to screen the specimen for the presence of Prilocaine. The combination of UPLC® and TOFMS allows for an untargeted, general screening methodology with much greater sensitivity and specificity/selectivity than more traditional methods (Gas Chromatography with Mass Spectrometry, High Performance Liquid Chromatography with Ultraviolet Detection, Immunoassay, and Thin Layer Chromatography) of screening postmortem specimens for drugs of abuse, therapeutic drugs, and other chemicals. The UPLC®/TOFMS could be considered a standalone qualitative instrument (if only qualitative results are required), but due to the forensic nature of our laboratory, all presumptive positive results from the preliminary screening assay *must* be confirmed by using a separate aliquot of the specimen and analyzing it via an alternate methodology.

In order to confirm the presumptive positive result from the UPLC®/TOFMS, a novel UPLC®/MS/MS method was developed for the analysis of Prilocaine. A complete method validation was performed for this new analysis. This method proved suitable for forensic toxicological purposes. Further investigation needs to be completed in order to add the metabolite, o-toluidine, to the analysis. Any future presumptive positive results for Prilocaine from the general unknown screening assay will be confirmed by this method.

## Acknowledgements

The authors of the presentation would like to thank Dr. Michael Evans, Dr. Andrea Terrell, and Dr. Scott Kriger for their support of this project and technical assistance.

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