A Rapid, General Unknown Screen in Blood by Ultra Performance Liquid Chromatography – Time of Flight Mass Spectrometry (UPLC-TOF)

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Abstract

Our objective was to develop a novel general unknown screening method for the simple and rapid analysis of drugs of abuse and therapeutic drugs in whole blood, serum and plasma.

Commonly used methods for general unknown screening in clinical and forensic laboratories include immunoassays, GC/MS, and HPLC-Diode Array Detector. Limitations of these techniques include long analytical run times, inadequate sensitivity, high specimen volume requirements and high reagent costs. Our method provides a sensitive, specific, rapid and cost effective solution for screening over 345 drugs using a simple protein precipitation extraction followed by UPLC-TOF analysis.

A 250 µL aliquot of specimen was precipitated with 1.0 mL of acetonitrile containing Proadifen as an internal standard. Specimens were vortexed, centrifuged, and the supernatant was transferred into a 96-well plate. Chromatographic separation was performed at 30˚C on a Waters ACQUITY UPLC system, followed by mass spectrometric analysis on a Waters LCT Premier XE Time of Flight mass spectrometer. A Waters Acquity UPLC HSS T3, 2.1 x 100 mm, 1.8 µm particle size column was used. Mobile phases consisted of Solvent A:0.05% Formic Acid in DI water and Solvent B: 0.05% Formic Acid in Optima grade Methanol. Initial mobile phase composition was 90%A:10%B. Following a short hold, a linear gradient was employed with a final mobile phase composition of 5%A:95%B. The total run time was 8 minutes per specimen. Scans were completed in both positive and negative electrospray ionization modes to identify both acidic and basic compounds. A low voltage scan was used for parent mass identification and a high voltage scan was used to identify mass fragments by in-source collision induced dissociation (CID). To extend the linear range, dynamic range enhancement (DRE) was used. Real time accurate mass data was acquired by reference to an independently sampled reference material (Leucine Enkephalin, [M+H] = 556.2771 amu). Validation experiments included an extensive comparison of results from our historical screening methods (ELISA & GC/MS) with the UPLC-TOF method.

A simple protein precipitation method was developed to screen for drugs of abuse and therapeutic drugs using UPLC-TOF mass spectrometry. The analytical run time was reduced from 26 minutes using GC/MS to 8 minutes using UPLC-TOF. A larger library could be developed on the TOF allowing us to expand the panel of drugs in our comprehensive general unknown screen from 120 analytes (GC/MS) to 345 analytes (UPLC-TOF). The new method also allowed us to eliminate 11 of 13 ELISA assays. A comparison study (GC/MS + ELISA vs. UPLC-TOF) demonstrated superior results using the TOF method. Detection limits varied for all major analytes; however the limits are appropriate for clinical and forensic use. The UPLC-TOF method has an expanded library of analytes, exceptional sensitivity and specificity, a much simpler specimen preparation step, and a faster analytical run time allowing us to cut our analysis time for a batch of 30 samples from 13 hours to 4 hours.

Introduction

Screening of specimens is used to evaluate possible accidental or deliberate overdose or poisoning of an individual. In many circumstances, it is undertaken to determine if drugs and/or chemicals are a contributing factor in the cause or manner of a death. Screening can also be completed for drug impairment cases or it can be undertaken on other types of specimens, such as powders or liquids of unknown origin. A screening analysis only detects drugs and/or chemicals; it is not a truly quantitative test. In order to determine if the specimen is truly positive for a drug and/or chemical detected on a screening analysis, a secondary or confirmatory analysis must be completed.

Traditionally, in the modern toxicology laboratory, a specimen was screened for drugs of abuse and therapeutic drugs by Enzyme Linked Immunosorbent Assay (ELISA) and Gas Chromatography with Mass Spectrometry (GC/MS). These instruments have done well in the past, but they have limitations.

ELISA has limitations of specificity and cross reactivity. The lack of specificity of ELISA can be seen with the many false positive results it yields. Since this type of methodology functions off an antigen/antibody reaction, an analyte with a very similar structure as the target class of analytes can bind and create a false positive result. This scenario was often seen with ELISA assays detecting tricyclic antidepressants, opiates, and amphetamines. This lack of specificity creates a problem for a toxicology laboratory by increasing the number of specimens that needlessly undergo confirmatory analysis, which result as negative for the target analytes.

Cross reactivity is another problem with using ELISA. This type of methodology uses an analyte as a target compound. Other analytes in the same drug class have a cross reactivity rate at which they will bind to the antibodies immobilized on the plate. This cross reactivity rate is dependent on the reagent or test kit. If the rate for a particular analyte is greater than one hundred percent, then the analyte has a lower limit of detection than the target compound. If the rate for a particular analyte is less than one hundred percent, then the analyte has a higher limit of detection than the target compound. This limitation can lead to a laboratory not being able to reach a needed detection limit of a specific analyte.

Two of the many problems associated with GC/MS are its lack of sensitivity and its inability to detect thermolabile and polar analytes. Lack of sensitivity of a method allows for false negative results, which are not acceptable in any toxicology laboratory. Due to this condition, many laboratories choose to screen for the typical or routine drugs of abuse by ELISA, and use a GC/MS methodology for the analytes not having ELISA reagent kits.

Thermolabile analytes, by definition, are very unstable at high temperatures; high temperature is fundamental in GC/MS. Polar and non-volatile analytes are also not suited for GC/MS analysis. In order to confidently detect these types of analytes, the specimen must be prepared through a rather long and tedious extraction method.(solid phase extraction, chemical derivatization, etc.) or 'clean up', which results in higher operating costs for a laboratory through consumables and a chemist's time.

Cost is a shared limitation with ELISA and GC/MS methodologies. Each ELISA reagent kit costs a substantial amount of money. In our laboratory, we were spending approximately thirteen dollars per specimen for our ELISA analyses. As mentioned above, the use of the GC/MS requires an extensive specimen 'clean-up' using solid phase extraction or something similar. In our laboratory, we were spending approximately $1.15 for each specimen for the solid phase extraction cartridge.

Ultra Performance Liquid Chromatography (UPLC®) and Time of Flight Mass Spectrometry (TOFMS) can alleviate the aforementioned problems. 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 unrivaled sensitivity and specificity, with accurate mass detection to four decimal places. The mass range (up to 30,000 m/z) of the time of flight mass spectrometer is also unequaled. 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, with ions of lighter masses traveling faster and reaching the detector in a shorter period of time. Ions of heavier masses travel slower and reach the detector in a longer period of time.

The objective of this project was to develop a new, **rapid** screening method for specimens using the novel approach of UPLC® and time of flight mass spectrometry. At the time of development, **no** toxicology laboratory in the United States of America has tried to implement this instrumentation for use in screening of whole blood specimens for drugs of abuse and therapeutic drugs.

Methods of Analysis

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, 1.8 µm particle size, held at 30 ºC. A gradient elution (Table 1) 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.

Table 1: The Acquity UPLC® Gradient

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

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 and a high voltage scan (Aperture voltage = 60) was used for in-source collision induced dissociation (CID) of the parent mass providing fragmentation. 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. ChromaLynx, version 4.1, is available for deconvolution of chromatographic data and parent mass fragmentation analysis through spectral library matching and exact mass scoring.

Extraction Procedure

A 250 µL aliquot of specimen (whole blood, plasma, serum, vitreous fluid, tissue, gastric contents, or non-routine matrices) was precipitated/diluted with Acetonitrile containing an internal standard (Proadifen, 10 ng/mL). The specimen was vortexed and centrifuged. The supernatant was placed into a 96 well plate or a glass autosampler vial and injected on the instrument.

Analyte Library

The analyte library consisted of 345 drugs, metabolites, and other chemicals. Whenever the laboratory receives a new reference standard, an entry is added to the accurate mass library. The method consisted of all of the common drugs of abuse and therapeutic or prescription drugs, along with many of the more esoteric compounds. All major drug classes are represented. The following list details some of the more esoteric analytes that are included in the method:

Anesthetics – Benzocaine, Etomidate, Mepivacaine, Procaine, Ropivacaine

Antibiotics – Azithromycin, Ceftriaxone, Ciprofloxacin, Erythromycin, Levofloxacin

Anticonvulsants – Gabapentin, Lamotrigene, Pregabalin, Valproic Acid, Zonisamide

Antidepressants – Citalopram, Duloxetine, Mirtazapine, Sertraline, Venlafaxine

Antihistamines – Brompheniramine, Hydroxyzine, Loratadine, Meclizine, Triprolidine

Antipsychotics – Aripiprazole, Clozapine, Quetiapine, Risperidone, Ziprasidone

Barbiturates – Allobarbital, Barbital, Hexobarbital, Mephobarbital, Methohexital

Benzodiazepines – Bromazepam, Demoxepam, Estazolam, Flurazepam, Triazolam

Cardiovascular – Amiodarone, Diltiazem, Furosemide, Metoprolol, Warfarin

Hallucinogens – LSD, Mescaline, PCP, Psilocin, Salvinorin A

Insecticides – Aldicarb, Bendiocarb, Carbaryl, Carbofuran, Methomyl

Muscle Relaxants – Baclofen, Metaxalone, Methocarbamol, Rocuronium, Tizanadine

NSAIDs – Etodolac, Flurbiprofen, Ketorolac, Oxaprozin, Piroxicam

Opioids – Alfentanil, Buprenorphine, Meperidine, Remifentanil, Sufentanil

Sedatives and Hypnotics – Ketamine, Ramelteon, Zaleplon, Zolpidem, Zopiclone

Steroids – Boldenone, Fluoxymesterone, Methandrostenolone, Nandrolone, Stanozolol

Stimulants – Benzphetamine, MDMA, Methcathinone, Methylphenidate

Others – Atomoxetine, Brodifacoum, Ropinirole, Sildenafil, Tadalafil

The accurate mass library is **not limited** to analytes that are in the library. Any theoretical accurate mass can be extracted from the total ion chromatogram; therefore, true unknown analysis can be completed making the library literally limitless, unlike the severe limitations seen in many of the more recently developed LC/MS/MS screening applications (MRM functions and dwell time issues).

Accurate Mass Capabilities

Accurate mass measurements within 1 – 5 ppm (parts per million) error are routinely made with this method and instrumentation. Figure 1 shows the extracted ion spectrum of a patient specimen that screened positive for Levetiracetam (Keppra®), a medication used to treat epilepsy and neuropathic pain. The theoretical accurate mass ([M+H]+) for Levetiracetam is 171.1134 Daltons. The calculated accurate mass is 171.1132 Daltons, which constitutes a -1.16 ppm accurate mass error. Any mass error less than 10 ppm is considered valid. Also, fragmentation of the parent mass is seen due to in-source collision induced dissociation (CID).

Figure 1. Extracted Ion Spectrum of Levetiracetam

Table 2. Calculated Accurate Masses from Replicate Injections

Analyte	Theoretical Accurate Mass	Injection	Calculated Accurate Mass	Mass Error (ppm)
Alprazolam	300.0907	1	300.0898	-2.99
		2	309.0907	0.00
		3	309.0909	0.66
		4	309.0915	2.66
		5	309.0911	1.29
Fentanyl	337.2280	1	337.2280	0.00
		2	337.2276	-1.18
		3	337.2275	-1.48
		4	337.2272	-2.37
		5	337.2275	-1.47
Zolpidem	308.1763	1	308.1766	0.97
		2	308.1762	-0.32
		3	308.1774	3.56
		4	308.1765	0.64
		5	308.1770	2.27

Replicate injections (5) were made of 3 patient specimens containing positive screening results for 3 common analytes in our laboratory. The results detailed in Table 2 show that the accurate mass capabilities of the instrument are routinely within 5 ppm mass error.

Dynamic Range Enhancement (DRE™)

Time of flight mass spectrometers have been known to have a limited dynamic range. The Dynamic Range Enhancement (DRE™) feature allows for greater semiquantitative capability by defocusing the ion beam by applying a high voltage to the mass spectrometer's Z-focusing lens and reducing the intensity of ions that are introduced into the mass spectrometer; therefore, extending the linear range of most analytes by four orders of magnitude. Without DRE™, most analytes at overdose/lethal/toxic concentrations will saturate the instrument's detector. Figures 2 and 3 and Table 3 detail the differences in linearity and accurate mass of Risperidone when DRE™ is not used and when it is used. When DRE™ is utilized, the linear range of Risperidone is 10 ng/mL – 5,000 ng/mL; whereas, when DRE™ is not utilized, Risperidone saturates the detector at some point between 250 and 500 ng/mL, making the linear range 10 – 250 ng/mL. The [M+H]+ of Risperidone is 411.2196 Daltons.

Table 3: Accurate Mass Error, Selected Calibration Curve Points

Concentration (ng/mL)	DRE Utilized (Yes or No)	Acquired Accurate Mass [M+H]+	Accurate Mass Error (PPM)
10	No	411.2196	0.0
10	Yes	411.2196	0.0
50	No	411.2195	0.2
50	Yes	411.2193	0.7
500	No	411.2150	11.1
500	Yes	411.2190	1.4
2000	No	411.2010	45.0
2000	Yes	411.2185	2.6
5000	No	411.1938	62.7
5000	Yes	411.2170	6.3

Figure 2. Linearity without using DRE™

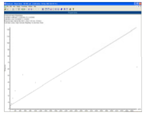
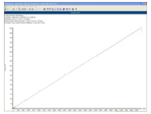


Figure 3. Linearity with using DRE™



Case Study – Determination of 'True' Unknown Analyte

History

A male is found deceased on a farm, outside of a barn. A puncture wound is seen on the decedent's anterior wrist. A syringe (with a bent needle) is found in close proximity to the victim. The syringe and the deceased's postmortem specimens (blood, urine, vitreous fluid) were all sent to the laboratory for screening.

Methods and Results

The syringe was rinsed with Methanol:DI Water [50:50]. A 200 µL aliquot of the rinse was diluted 1:10,000 using Methanol:DI Water [50:50].

The syringe rinse and postmortem specimens were run according to the outlined screening method. All specimens were precipitated/diluted with 1 mL of acetonitrile containing an internal standard, vortexed, and centrifuged. The supernatant was placed into a 96-well plate for injection on the instrument.

The specimens were negative for all drugs of abuse and therapeutic drugs contained within the accurate mass library. An analyte peak ([M+H]+ = 869.57) at 3.57 minutes was observed in the syringe rinse and postmortem specimens. Research was conducted to find a drug or chemical that had an [M+H]+ equal to 869.57 Daltons. It was found that a macrolide antibiotic drug for use in cattle, sheep, and goats (Tilmicosin, trade name Micotil®) fit the requirement. A reference standard was obtained from a supplier and spiked in blood.

Figures 4 and 5depict the extracted ion chromatogram of the Tilmicosin reference standard (Theoretical [M+H]+ = 869.5739 Daltons), along with the chromatogram of the internal standard. The extracted ion spectrum of Tilmicosin is also shown.

Figure 4

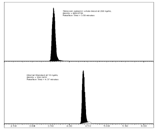
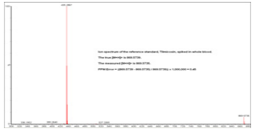


Figure 5



Figures 6 and 7 depict the extracted ion chromatogram of the postmortem whole blood specimen ([M+H]+ = 869.5760 Daltons), along with the chromatogram of the internal standard. The extracted ion spectrum of the peak at 3.57 minutes is also shown.

Figure 6

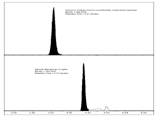
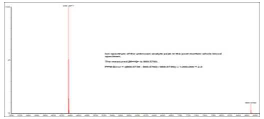


Figure 7



The peak from the reference standard and the syringe rinse share the same retention time and accurate mass. The specimen was confirmed positive for Tilmicosin by a secondary analysis (LC/MS/MS). It was concluded that the deceased man had accidentally injected himself with Tilmicosin and died. Tilmicosin is not for use in humans and is not to be injected intravenously in any living animal. Injecting the drug intravenously has proven to be fatal.

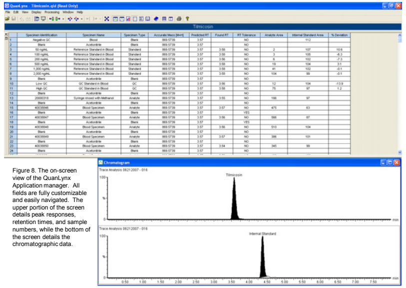


Figure 8. The on-screen view of the QuanLynx Application manager. All fields are fully customizable and easily navigated. The upper portion of the screen details peak responses, retention times, and sample numbers, while the bottom of the screen details the chromatographic data.

Application and Conclusion

A novel, rapid UPLC/TOFMS method was developed making AIT Laboratories the only laboratory in the United States of America to utilize the Waters Acquity UPLC® and LCT Premier™ XE in the realm of toxicology.

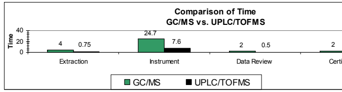
The new method has greater sensitivity and specificity than either the ELISA or the GC/MS method. As an example of sensitivity of the new method, the ELISA used Oxazepam at 50 ng/mL as the target compound for the benzodiazepine class of drugs. With the specific reagent kit, Alprazolam had a 70% cross reactivity rate. Therefore, the actual screening concentration of Alprazolam was 85 ng/mL. With the low detection limits achieved by the new method, Alprazolam is currently able to be screened at a cutoff of 2.5 ng/mL As an example of specificity, by switching from ELISA and GC/MS to the new method, the laboratory has decreased the amount of false positive tricyclic antidepressant screen results for specimens sent to confirmation by approximately 75%.

Due to the greater sensitivity, 11 of 13 ELISA assays have been replaced by the UPLC/TOFMS method of analysis. Each assay on the ELISA costs approximately $1 per specimen and by eliminating 11 of the assays, the laboratory is saving approximately $11,000 per month.

The new method allows for detection of polar and thermolabile analytes, which are not detected very well by a traditional GC/MS method. Due to the more consistent detection of these analytes, the laboratory's comprehensive drug panel has been expanded by approximately 200 analytes and will continue to be expanded whenever the appropriate confirmation assays can be developed. As an example, Citalopram was added to the panel in May 2007. Between January 1, 2006 and the date of addition, the laboratory extracted 26 postmortem specimens for confirmation of Citalopram. Because the analyte was not included in the laboratory's comprehensive drug screen panel, all of the specimens were directed analyses by the client. Between May 16, 2007 and May 16, 2008, the laboratory had detected more than 600 postmortem cases with positive screen results for Citalopram. 97.3% of those presumptive positive results have confirmed positive (greater than 10 ng/mL) by an alternate method.

The use of the UPLC® allows for shorter run times and shorter specimen preparation times. The GC/MS method of analysis consisted of a long and tedious solid phase extraction. Extraction time of a batch of specimens was approximately 4 hours. Injection to injection run time of one specimen on the GC/MS method was 26 minutes. Initial data review and quality assurance data certification time was approximately 2 hours for each process. The new method of analysis consisted of a simple protein precipitation/dilution with acetonitrile. Extraction time of a batch of specimens for the new method was 45 minutes. Injection to injection run time of one specimen on the UPLC/TOFMS method was 6.5 minutes. Initial data review and quality assurance data certification time was approximately 30 minutes and 45 minutes respectively.

Figure 9: Comparison of the Efficiency of Two Screening Methods



By using the UPLC/TOFMS method, we have eliminated the GC/MS method and 11 of 13 ELISA assays. Specimen preparation time has been decreased by 75%; specimen run time has decreased by 75%. Turn around time from screening to confirmation has decreased substantially. Between May 15, 2006 and May 15, 2007, 8,708 specimens were screened on the GC/MS utilizing the comprehensive panel, with greater than 500 matrix related failures. Between May, 16, 2007 and May 16, 2008, 10,880 specimens were screened on the UPLC/TOFMS utilizing the comprehensive panel, with only 39 matrix related failures. AIT Laboratories is saving approximately $13,000 (total) per month by using this method.

Table 4. Costs Savings Analysis

Assay	Cost	Calculation	Daily Savings	Weekly Savings	Monthly Savings	Annual Savings
Reagent Kit (ELISA)	$1.00	11 x $1 x 50 specimens	-$550	-$2,750	-$11,000	-$132,000
Extraction Cartridge (GC/MS)	$1.15	$1.15 x 50 specimens	-$58	-$290	-$1,160	-$13,920
Extraction Time (GC/MS)	$16.83	4 hours per batch of 50 specimens	-$67	-$335	-$1,340	-$16,080
Extraction Time (UPLC/TOFMS)	$16.83	0.75 hour per batch of 50 specimens	+$13	+$65	+$260	+$3,120
Total	-	-	-$662	-$3,310	-$13,240	-158,880

Overall, the UPLC/TOFMS method is much more cost effective and far superior to the combination of ELISA and GC/MS methods of analyses in all facets.

Acknowledgements

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