TAMPEREEN
AMMATTIKORKEAKOULU

TESTING OF AN LC-QTOF-MS METHOD FOR THE SIMULTANEOUS SCREENING AND QUANTIFICATION OF 100 DRUGS AND METABOLITES IN POST-MORTEM BLOOD SAMPLES

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ABSTRACT

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Testing of an LC-QTOF-MS Method for the Simultaneous Screening and Quantification of 100 Drugs and Metabolites in Post-Mortem Blood Samples

Bachelor's thesis 52 pages, Appendices 4 pages
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This bachelor's thesis was commissioned by Analytical Services International Ltd in the city of London, UK. The aim was to test a method developed Agilent Liquid Chromatography Time-of-flight Mass Spectrometer (LC-QTOF-MS) to detect and quantify drugs and metabolites in post-mortem blood samples as a part of the method development procedure in ASI. The main reason for this was to make 'full toxicology' analysis process faster and more accurate, to meet the required turnaround times. Previously, the 'full toxicology' analysis required several different analyses, with different instruments and the required turnaround time of an average of fifteen days was rarely achieved. At the beginning of the project the number of overdue cases was increasing every week.

The method was developed using an Agilent 1290 II liquid chromatography system equipped with a 6545 QTOF-MS. The instrument consisted of a liquid chromatography system, an ionization source, a quadrupole mass filter for fragmentation, a time-of-flight analyzer and a mass detector. In the liquid chromatography system, the compounds of interest were separated and then ionized as they entered the mass spectrometer via electrospray ionization (ESI). After this, the ions were sent through the quadrupole into the time-of-flight analyzer where separation took place based on the ions' mass-to-charge ratios. Finally, the ions arrived at the detector.

Implementation of the combined screening and quantification analyses via LC-QTOF-MS for both blood and urine specimens meant (i) more compounds could be identified than using the previous combination of assays, and (ii) fewer additional quantitative assays were required. The introduction of LC-QTOF-MS for carrying out 'full toxicology' analysis improved the turnaround times significantly due to the reduction in the number of unnecessary assays.

Keywords: time-of-flight, toxicology, drugs, cause of death, forensics, liquid chromatography, drug screen, quantitative analysis, mass spectrometry, blood sample

TIIVISTELMÄ

Tampereen ammattikorkeakoulu
Laboratorioalan koulutusohjelma

KOSONEN, SIIRI:
100 lääkeaineen ja metaboliatuotteen seulonta ja samanaikainen määrittäminen kuoleman jälkeisistä verinäytteistä korkean erotuskyvyn kvadrupoli-lentoaika-analysaattorilla

Opinnäytetyö, 52 sivua, liitteitä 4 sivua
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Opinnäytetyö tehtiin Analytical Services International Ltd -nimisen bioanalytiikan ja toksikologian laboratorion toimeksiantona Lontoossa, Iso-Britanniassa. Tarkoituksena oli testata menetelmää korkean erotuskyvyn kvadrupoli-lentoaika-analysaattorilla (LC-QTOF-MS) osana toimeksiantajan menetelmäkehitysprosessia. Tavoitteena oli nopeuttaa lääke- ja huumausaineiden seulontaa ja määritystä vainajanäytteissä. Aiemmin laajaan toksikologiseen analyysiin sisältyi useita analyyseja eri laitteilla ja menetelmillä, mikä teki tulosten raportoinnista annettujen aikarajojen sisällä miltei mahdotonta, ja alussa keskeneräisten tapausten määrä oli jatkuvassa kasvussa.

Menetelmäkehitykseen käytettiin nestekromatografista, ionilähteestä, kvadrupolista, törmäyskammiosta, lentoaika-analysaattorista ja detektorista koostuvaa Agilent 1290 II nestekromatografin ja 6545 QTOF-massaspektrometrin yhdistelmää. Nestekromatografisysteemissä yhdisteet eroteltiin toisistaan ja ionisoitiin sähkösumutustekniikalla (ESI). Syntyneet ionit ohjattiin kvadrupolin läpi lentoaika-analysaattoriin, jossa ionit eroteltiin niiden massa per varaus -suhteiden perusteella, jonka jälkeen ne saapuivat detektorille.

Lääke- ja huumausaineiden samanaikainen seulonta ja määrittäminen LC-QTOF-MS laitteistolla veri- ja virtsanäytteistä mahdollistivat aiempaa suuremman näytemäärän analysoinnin kerralla. Tämän lisäksi se nopeutti huomattavasti tulosten saantia vähentämällä tarpeettomien analyysien määrää.

Asiasanat: toksikologia, seulonta, lääkeaineet, huumausaineet, lentoaika-analysaattori, nestekromatografia, massaspektrometria, kvantitatiivinen analyysi, verinäyte, vainajanäytteet, kuolinsyy

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ABBREVIATIONS AND TERMS

ADHD	Attention deficit hyperactivity disorder
API	Aatmospheric pressure ionization
ASI	Analytical Services International Ltd
BZP	1-benzylpiperazine
CBD	Cannabidiol
Deuterium, D	Stable isotope of hydrogen used as a marker on drugs in the internal standard
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine
ESI	Electrospray ionization
Fragment ion	Fragment ion that results from dissociation of a precursor ion
Full Toxicology Analysis	Includes screening and quantitation of the most common drugs and metabolites taking the medical history of the deceased and the circumstances of death into consideration
GABA	Gamma-aminobutyric acid
GCMS	Gas chromatography-mass spectrometry
LC	Liquid chromatography
LC-MS or HPLC-MS	Liquid chromatography-mass spectrometer
LC-QTOF-MS	Liquid chromatography quadrupole time-of-flight mass spectrometer
Legal highs	Previously legal substances that mimic the effects of illegal drugs, but are banned in the UK since 2016
mCPP	meta-chlorophenylpiperazine
MDA	Methylenedeoxyamphetamine
MDEA	Methylenedioxy-N-ethylamphetamine
MDMA	3,4- methylenedeoxymethamphetamine or ecstasy
MDPV	3,4-methylenedioxypyrovalerone
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry. A technique to break selected ions down into fragments which will then reveal aspects of the precursor ions chemical structure
m/Z	Mass-to-charge
Neurotransmitters	Chemical messengers in the body

NSAID	Non-steroidal anti-inflammatory drug
NPS	New psychoactive substances
Precursor ion	An ion of a particular mass-to-charge ratio
SNRI	Selective serotonin-norephedrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TFMPP	trifluoromethylphenylpiperazine
Turnaround time	Number of days counted from the arrival of samples to the day the results are reported. Usually 15 working days
6-MAM	6-monoacetylmorphine
Δ9-THC	Delta-9-tetrahydrocannabinol

1 INTRODUCTION

The Analytical Services International Ltd (ASI) is a leading bioanalysis and toxicology laboratory located in London, United Kingdom. ASI receives samples from mortuaries and hospitals mostly from within and sometimes outside of the United Kingdom. These specimens mostly consist of plain and preserved blood, urine, vitreous humour and stomach contents collected from people who have deceased possibly under somehow suspicious circumstances and whose cause of death is unascertained. The number and type of samples depend on their availability and the circumstances of death. In the absence of blood availability, the samples can be sections of tissue (e.g. liver or muscle) or the specimen may be an unidentified object found in the throat of the deceased. The clients provide the samples and request either a full toxicology analysis or names the drugs they wish the samples to be analyzed for.

The aim of this thesis was to test a newly developed method for the quadrupole time of flight analyzer as a part of the method development procedure at ASI. The objective was to shorten the toxicology routine analysis making it possible to obtain results within given turnaround times.

This project was carried out under the supervision of Dr. Lewis Couchman, the Facility Director at ASI. With the first-hand knowledge of the toxicologists who work with these cases daily it was possible to provide more accurate content for this thesis. A temporary full-time contract of employment was signed with ASI as until the 24th of August 2018. The project was carried out alongside the regular tasks of a laboratory technician.

2 THEORY

2.1 Forensic Toxicology

Forensic toxicology is a part of the science of pharmacology which focuses on the effects of drugs and poisons on human beings. The main interest is the effect of a drug or poison in the impairment or death of a person. Many of the cases received by forensic toxicologists are cases of driving under the influence of alcohol. As drinking and driving is illegal, forensic toxicologists are called upon to determine the concentration of alcohol present in the body and the effects on the driver. When drugs or poisons are involved in the case, forensic toxicologists get involved when a death has occurred helping to determine the cause and manner of death. The samples are screened for drugs and poisons and any substance of interest are quantitated to determine the role drugs or poisons played in the cause of death. (Houck & Siegel. 2015.)

2.2 Full Toxicology Analysis

The full toxicology analysis in forensic toxicology is something that has been modified over the years to keep up with today's requirements. It consists of a screening of drugs followed by a quantification of the drugs detected. There are, however, hundreds of drugs and toxins that may play a role in the cause of death, so the full toxicology analysis must be set to reasonable limits, otherwise the analysis will be too time-consuming and therefore not very cost-effective for the company. The limits are determined by the demand for certain analyses and the frequent appearance of some substances of interest in post-mortem samples. This means a full toxicology analysis in any forensic toxicology laboratory would at least include prescription drugs and most of the licit and illicit drugs, and alcohol (ethanol). An example of a full toxicology analysis is presented in Appendices 1 and 2. Sections from 2.2.1 to 2.2.17 introduce examples of drugs that blood and urine samples can be analysed for as a part of a full toxicology analysis. A full toxicology analysis, however, does not necessarily limit to these substances.

2.2.1 Anaesthetics

Simply put, anaesthesia means loss of sensation. Anaesthetic drugs are used during surgical operations and tests to numb specific areas in the body or to induce sleep to prevent pain and discomfort. There are anaesthetics that can be used locally on a limb for example while the patient is fully conscious. General anaesthetics render the patient fully unconscious, which is useful in more serious operations. Anaesthetics work by stopping certain nerve signals from reaching the brain. (NHS UK. 2018.)

2.2.2 Antiarrhythmics

Antiarrhythmics are classified based on their electrophysiological effect on the myocardium (the middle muscular layer of the cardiac wall). Antiarrhythmic drugs include calcium channel blockers, beta blockers, lidocaine, amiodarone and cardiac glucosides. These drugs also have other medical uses. (Amboss. 2018.)

Antiarrhythmic medications may increase mortality especially in patients who suffer from a structural heart disease. They are also are associated with severe adverse effects, mostly because of the potentially proarrhythmic effect they may have on the myocardium, which means they may cause emergence of further arrhythmias. This is why the use of antiarrhythmics must be well monitored. (Amboss. 2018.)

2.2.3 Antidepressants

Antidepressants are a type of medication used to prevent or treat clinical depression. It is believed that antidepressants work by increasing levels of neurotransmitters (chemical messengers) such as noradrenaline and serotonin in the brain and that way can improve mood and emotion. Increased levels of these neurotransmitters can also disrupt nerve signals which is why antidepressants can also be used to relieve chronic pain. Usually, antidepressants are used in combination with therapy as the drugs alone do not treat the root cause for the depression. (NHS UK. 2018.)

While most people benefit from taking antidepressants, the drugs may not be as efficient in cases of mild depression as they are in more severe cases. There can also be side effects to taking antidepressants, but they generally improve with time. An overdose of antidepressants can cause symptoms like tremor, uncontrolled movement of the eyes, feeling agitated and severe muscle tension. (NHS UK. 2018.)

Cyclic antidepressants

Tri- and tetracyclic antidepressants are among the earliest developed antidepressants. They have lately been replaced with drugs with fewer side-effects. They are designated tri- or tetracyclic based on the number of rings in their chemical structure, tri standing for three and tetra standing for four. They work by impacting neurotransmitters used to communicate between brain cells, helping to relieve depression by regulating mood. (Mayo Foundation of Medical Education and Research, MFMER. 2018.) According to the NHS UK symptoms of cyclic antidepressants include excitability, enlarged pupils, dryness of mouth, irregular or rapid heartbeat and lowered blood pressure which may lead to fainting.

Selective serotonin and serotonin-norephedrine reuptake inhibitors

Selective serotonin (SSRI) and serotonin-norephedrine reuptake inhibitors (SNRI) are a newer type of antidepressants used to treat conditions such as obsessive-compulsive disorder and anxiety disorder. SSRIs relieve depression by increasing levels of serotonin in the brain. Serotonin is one of the neurotransmitters that carry signals between brain cells. SSRIs make more serotonin available by preventing the reabsorption of serotonin in the brain. SSRIs are called selective because they appear to primarily affect serotonin, not other neurotransmitters. (MFMER. 2018.) SNRIs affect two important neurotransmitters – serotonin and norephedrine and are also sometimes called dual reuptake inhibitors or dual-acting antidepressants (Healthline Media. 2018).

Potential health risks to taking SSRIs and SNRIs include serotonin syndrome, when the serotonin levels become too high and hyponatremia when the drug causes severe fall in

sodium concentrations in the body. In some rare cases antidepressants may cause suicidal thoughts. (NHS UK.)

2.2.4 Antiepileptics

Antiepileptics are the main form of treatment for people suffering from epilepsy, and up to 70% of them could have full control over their seizures with antiepileptic drugs. (Epilepsy Society. 2018.) According to The Royal Children's Hospital in Melbourne antiepileptic drugs do not cure epilepsy, but rather prevent epileptic seizures. People with epilepsy are prescribed antiepileptic medications to decrease the number, severity and shorten the duration of the seizures.

For a brain to function normally there needs to be fluent communication between millions of nerve cells. At any one time there are nerve cells either resting, exciting or inhibiting other nerve cells. The antiepileptic drugs work different ways either by decreasing excitation or by enhancing inhibition. (The Royal Children's Hospital Melbourne. 2018.)

2.2.5 Antihistamines

Antihistamines are drugs often used for relieving symptoms of allergies. These include hay fever, conjunctivitis (pink eye), hives and reactions to insect stings and bites. They can also be used for easing motion sickness and insomnia. There are many antihistamines that can be bought over the counter in shops and pharmacies, but some are only available with prescription. Antihistamines are mainly divided into two main groups – older antihistamines that causes drowsiness, and newer antihistamines that do not. Depending on the type of antihistamine, the side-effects may include drying of mouth, blurring of vision, sleepiness, nausea and headaches. (NHS UK. 2018.)

2.2.6 Antimalarials

Antimalarial drugs are used to either treat malaria, or to prevent a person from getting it. Malaria is a serious mosquito-borne disease caused by parasites of the *Plasmodium* species. It causes high fever, chills and possibly life-threatening flu-like symptoms if not treated quickly. There are four main drugs used to treat malaria – quinoline-related compounds, artemisinin derivatives, antimicrobials and antifolates. The drugs inhibit growth by concentrating within acid vesicles of the parasite, increasing the internal pH of the organism. They also inhibit the metabolism of the parasites and prevent them from utilizing hemoglobin in the blood. (Herchline. 2018.)

2.2.7 Antipsychotics

Antipsychotics, also known as neuroleptic drugs, are used to reduce dopaminergic neurotransmission to treat psychiatric conditions such as dementia, sleeping disorders, mania, hallucinations and schizophrenia. Antipsychotic drugs are divided into typical and atypical antipsychotics. (Kratzsch, Peters, Kraemer, Weber & Maurer. 2003.)

According to the Alzheimer's Society in United Kingdom the possible side effects of antipsychotic drugs include increased risk of blood clots, stroke and infections, swelling of the ankles, drowsiness and increased risk of falling. All antipsychotics are dopamine D2 antagonists but most of them also block other monoamine receptors. Clozapine also blocks D4 receptors.

Older antipsychotic drugs, often referred to as typical or classical antipsychotics were first synthesized in the 1950s. Possible side effects of typical antipsychotics are stiffness and shakiness, feeling slow, restlessness, breast swelling and tenderness. They can also affect blood pressure, and cause dizziness. (Rang, Dale, Ritter & Moore. 2003.)

Newer antipsychotic medications, often referred to as atypical or second-generation antipsychotics, block dopamine receptors but less so than the older drugs. However, they still have many of the same side effects as the older ones. Side effects typical to newer antipsychotics include weight gain and increased risk of developing diabetes. On the other

hand, they are less likely to cause stiffness of limbs than the older drugs. (Rang et al. 2003.)

2.2.8 Barbiturates

Barbiturates are central nervous depressants used to treat headaches, seizures and insomnia. They relax muscles by reducing activity of nerves and can cause lowered heart rate and blood pressure. All barbiturates affect gamma-aminobutyric acid (GABA), a brain chemical nerves use to communicate with one another. Common side-effects of barbiturates include dizziness, headache, nausea, abdominal pain and sedation. Barbiturates can also be addictive or interact with other medications by accelerating breakdown leading to lowered effectiveness of the other drugs. Concomitant use of barbiturates and other nervous system depressants can lead to excessive sedation, lethargy and even coma or death. (MedicineNet, Inc. 2018.)

2.2.9 Benzodiazepines, non-benzodiazepines and benzodiazepine antagonists

Benzodiazepines

Benzodiazepines are heterocyclic compounds which consist of a benzene and a diazepine ring, with four main substituent groups that can be modified without loss of activity. They act selectively on gamma-aminobutyric acid A ($GABA_A$) receptors which mediate fast inhibitory synaptic transmission in the central nervous system. Benzodiazepines intensify the response to GABA by facilitating the opening of chloride channels. The main effects of benzodiazepines include reduction in aggression and anxiety, reduction of muscle tone and coordination, sedation, inhibition of convulsions and anterograde amnesia (inability to store and retain new information). (Rang et al. 2003.)

Benzodiazepines are excreted from the body in urine as glucuronide conjugates. Signs of benzodiazepine poisoning include difficulties of speech, shallow breathing, coordination difficulties, drowsiness and uncontrollable movement of the eyes. (NHS, UK. 2018.)

Non-benzodiazepines

Non-benzodiazepines are sometimes also referred to as Z-drugs. Z-drugs are a group of psychoactive drugs that are very similar to benzodiazepines and their pharmacodynamics are almost entirely the same which is why nonbenzodiazepines employ similar benefits and side-effects as benzodiazepines. (Rang et al. 2003.) According to Olson's online publication (2008) in the Australian Prescriber the Z-drugs have been promoted as being safer than benzodiazepines, and in many countries, they are the most widely prescribed drugs for insomnia.

The Z-drugs are agonists at the same $GABA_A$ receptor. In treatment of insomnia they possess a shorter half-life and duration of action and cause less residual effects during daytime hours, making them more clinically attractive than benzodiazepines. Poisoning with Z-drugs involves predominantly sedation and coma. (Gunja. 2013.)

Competitive benzodiazepine receptor antagonists

Competitive benzodiazepine receptor antagonists like flumazenil are used to reverse the sedative effects of benzodiazepines during surgeries and other medical procedures. It can also be used as an antidote in cases of benzodiazepine overdose. (Rang et al. 2003.)

2.2.10 Cannabinoids

Cannabinoids are compounds naturally occurring in the *Cannabis sativa* plant. There are over 480 different compounds present in the plant but only about 66 of them are considered cannabinoids, the most well-known of them being delta-9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD). (Mandal. 2018.)

Cannabinoids interact with specific cannabinoid receptors on the surface of cells found in different parts of the nervous system. Different cannabinoids are differentiated based on how psychoactive they are. Nowadays medicinal use of cannabis is approved in some countries under supervision for the treatment of anorexia, nausea and neuropathic pain

for example. Most common side-effects of cannabinoids dizziness and tiredness. (Grotenhermen & Müller-Vahl. 2012.)

2.2.11 Ethanol

Ethyl alcohol, also known as ethanol or grain alcohol is the principal ingredient in alcoholic beverages. It dissolves well in water, which is why it is also an ingredient in various products from cosmetics to fuel. Ethanol is a natural byproduct of plant fermentation. (ChemicalSafetyFacts.org. 2018.) Ethanol is a substance commonly analyzed in forensic toxicology in cases of drinking and driving.

2.2.12 Markers of ketoacidosis

According to the American Diabetes Association (2016) diabetic ketoacidosis (DKA) is a state where acids build up in the blood. It may occur when blood sugar levels remain too high for too long and could be life-threatening. DKA usually happens because the body does not produce enough insulin and therefore cannot utilize the sugar in the blood for energy. Instead, the body fuels itself with the fat in the body producing acids called ketones. Excess ketones build up in the blood and disrupt its chemical balance and could throw off the entire system.

DKA is most likely to happen to people with type 1 diabetes where the body does not have any insulin. It is quite rare, yet possible in people with type 2 diabetes. Especially older people with type 2 diabetes are more likely to have a different condition with similar symptoms called hyperosmolar hyperglycemic non-ketotic syndrome, which can lead to severe dehydration. (American Diabetes Association. 2016.)

People diagnosed with diabetes have ways to measure their blood sugar and the level of ketones in the body. There are also warning signs which indicate high blood sugar such as dry mouth, feeling really thirsty or urinating frequently. (American Diabetes Association. 2016.) Also, acetone, one of the ketones produced in the body, can make the breath smell strongly of nail polish.

2.2.13 Narcotic analgesics

Narcotic analgesics, also called opiates, opioid analgesics or narcotics, are medications used to relieve moderate or severe acute or chronic pain. They work by binding to opioid receptors mostly in the brain and spinal cord but are also located in other parts of the body such as lungs or stomach. (Drugs.com. 2018.)

Opiates are naturally occurring alkaloids found in the poppy plant *Papaver somniferum*. (Pappas, S. 2017). Opioids are synthetic drugs or endogenous compounds which bind to the opioid receptors in the body and are subject to abuse for their effects. Diacetylmorphine (heroin) abuse is the most common. (Rang et al. 2003.) Due to the short half-life of heroin its metabolites 6-monoacetylmorphine (6-MAM) and morphine are quantified. 6-MAM is a metabolite specific for heroin only and is therefore used as marker for heroin use. (von Euler, Villén, Svensson & Ståhler. 2003.)

Narcotic analgesics induce relaxation, drowsiness and sedation and alleviate anxiety. This may impart a state of enhanced mood or euphoria and is also why the drugs are subject to abuse. Opioids produce a physical dependence and withdrawal symptoms and the body develops a tolerance to the drug in habitual use over time. (Encyclopedia Britannica. 2018.) Symptoms of opioid poisoning include shallow breathing, drowsiness and narrowing of pupils (NHS UK. 2018).

2.2.14 Simple analgesics

Non-opioid analgesics, also known as simple analgesic medications include non-steroidal anti-inflammatory drugs (NSAIDs) and paracetamol. Most of them relieve fever and have anti-clotting properties. Anti-inflammatory medications relieve pain and inflammation within the body by decreasing the production of pro-inflammatory chemicals called prostaglandins. Paracetamol also reduces the production of prostaglandins but has fewer adverse effects. (Virtual Medical Centre. 2017.)

According to the Virtual Medical Centre (2017) the common side-effects of NSAIDs include diarrhea, ulceration or bleeding of the stomach or intestines and high blood pressure. Paracetamol may cause dyspepsia or allergic reactions and blood disorders.

2.2.15 Piperazines

Piperazines are frequently sold as ‘ecstasy’ under generic names such as ‘social tonics’, ‘pep pills’ or ‘party pills’. Some piperazines have been evaluated as potential therapeutic agents but were never brought to markets, while others have been described as ‘failed pharmaceuticals’ (King & Kicman. 2011). According to the United Nations Office on Drugs and Crime (2018) most of the piperazines act as central nervous system stimulants (2.2.16), but in some rare cases they can also act as opioids (2.2.13). Most common piperazines include 1-benzylpiperazine (BZP), meta-chlorophenylpiperazine (mCPP) and trifluoromethylphenyl piperazine (TFMPP).

2.2.16 Stimulants

Stimulant drugs, sometimes called ‘uppers’ are a class of substances which temporarily increase energy and alertness by increasing certain types of cell signaling and amplifying various physiologic processes throughout the brain and body. Many types of stimulant drugs are associated with heightened dopamine release resulting in a powerful sense of well-being and alertness. (Thomas, Villa, Crane, Watkins, Wagener & Brande. 2018.)

Stimulant drugs include prescription medications for the treatment of ADHD (attention deficit hyperactivity disorder) such as Adderall (amphetamine and dextroamphetamine) and Ritalin (methylphenidate). Stimulants also include illicit drugs like amphetamine, methamphetamine and cocaine. (Thomas et al. 2018.) The symptoms caused by an overdose of stimulant drugs such as ecstasy, cocaine or amphetamine include restlessness, anxiety, paranoia, hallucinations, chest pain, rapid or irregular heartbeat, and rapid breathing. (NHS, UK. 2018.)

2.2.17 Synthetic cathinones

Cathinone is a naturally occurring amphetamine found in the *Catha edulis* plant (khat). The drug causes stimulant effects such as improved mood and increased heart rate.

Synthetic cathinones, also known as ‘bath salts’, are chemically related to cathinone but are specifically produced for drug abusers as legal substitutes for cocaine, ecstasy and amphetamines (often referred to as legal highs). Unlike traditional bath salts for cosmetic use, these toxic bath salts have no legitimate use in bathing and are produced specifically for recreational use. (Baumann. 2014.) Legal highs, or substances that mimic the effects of illegal drugs, were banned by the government in United Kingdom in 2016 (GOV.UK. 2016).

Synthetic cathinones are considered as a part of the group of new psychoactive substances (NPS). The NPS are unregulated mind-altering substances with no legitimate medical use. A study in by Baumann et al. (Neuropsychopharmacology. 2013; 38(4): 552-62) found that a common synthetic cathinone known as MDPV (3,4-methylenedioxypyrovalerone) has an effect on the brain like that of cocaine but is at least 10 times more powerful. It is the most common synthetic cathinone found in the blood and urine of patients brought to emergency departments after taking ‘bath salts’. (Baumann et al. 2013.)

Typical effects of synthetic cathinones include increased sex drive, paranoia and hallucinations but also panic attacks, agitation and violent behavior. Synthetic cathinones may cause health issues like raised heart rate and blood pressure. The drugs can be addictive and often cause withdrawal symptoms such as depression, anxiety, insomnia, tremors and paranoia. (National Institute on Drug Abuse. 2018.)

2.3 LC-QTOF-MS

The liquid chromatography quadrupole time-of-flight mass spectrometer (LC-QTOF-MS) consists of a liquid chromatography system, an ionization source, a quadrupole mass filter, a high-resolution time-of-flight mass analyzer and a detector. Analytes are separated in the liquid chromatography system and ionized using electrospray ionization (ESI) in the ion source. To build spectral libraries, the quadrupole is used to select precursor ions that are fragmented in the collision cell into analyte-specific fragment ions. Compounds are identified by comparing the chromatographic retention times and the co-elution of the precursor and fragment ions with the MS/MS spectral libraries. Structure of LC-QTOF-MS is demonstrated in Figure 1. (Agilent Technologies, Inc. 2014.)

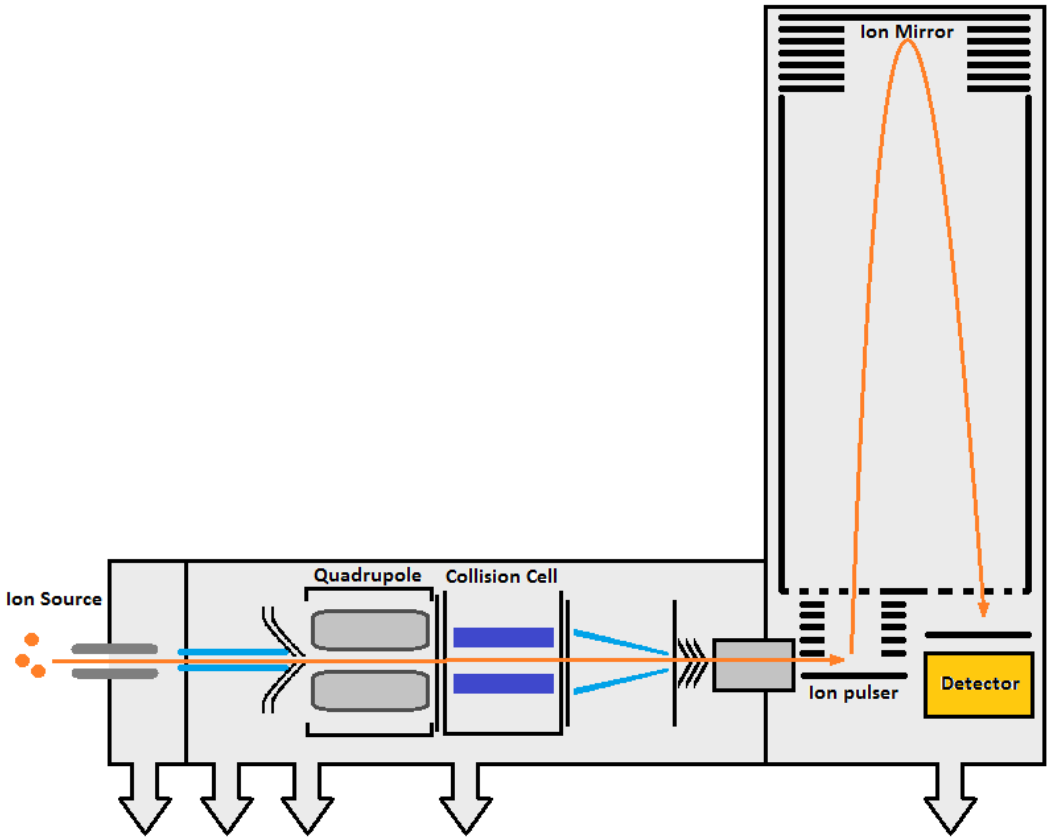

Figure 1. Schematic of LC-QTOF-MS. (Agilent Technologies, Inc. 2014.)

2.3.1 Liquid Chromatography

Liquid Chromatography (LC) is a technique used to separate components using a polar mobile phase and a non-polar stationary phase in a column. The higher affinity to the stationary phase a component has, the longer it will take for it to flow through the column. The time it takes for a component to flow through the column is called retention time. (Long. 2018.)

High-performance liquid chromatography (HPLC), also known as high-pressure liquid chromatography, is a more advanced type of liquid chromatography. It is used for several applications, pharmaceuticals for example, but is especially useful for low or non-volatile organic compounds. Compounds are determined based on their retention times. (Long. 2018.) The difference between HPLC and traditional LC is that HPLC operates in higher pressure, 50-350 bar, whereas the traditional LC relies on gravity. (Thermo Fisher Scientific Inc. 2016.)

2.3.2 Mass Spectrometry

Mass Spectrometry is an analytical method used to determine the *m/Z* ratios of ions. The mass spectrometer (MS) consists of an ion source, a mass analyzer and a detector. It ionizes chemical species, sorts the produced ions based on their *m/Z* ratios and then records the relative abundance of each ion type. The result is displayed in the form of a plot of ion abundance versus *m/Z* ratio. Each ion provides information concerning the nature and structure of its precursor molecule. In the spectrum of a pure compound, the molecular ion appears at the highest value of *m/Z* and gives the molecular mass of the compound. (Premier Biosoft. 2018.)

HPLC-MS (or LC-MS) is a combination of HPLC and MS. It involves separating mixtures based on their physical and chemical properties, identifying components within each peak and detecting them based on their mass spectrum. As a combination of two analytical methods LC-MS improves accuracy and reduces experimental error. (Long. 2018.)

2.3.3 Electrospray Ionization, ESI

Electrospray Ionization (ESI) is a so-called soft ionization technique and belongs in a group of methodologies known as atmospheric pressure ionization (API) techniques. It uses three important processes: production of charged droplets, desolvation of the droplets and production of gas-phase ions. (Taylor. 2012).

In ESI the liquid obtained from the liquid chromatography system is sprayed into small droplets using high voltage in atmospheric pressure. The ions or molecules are transferred into the gaseous phase before sampling into a mass analyser as ionized species. The ESI is suitable for the analysis of polar biomolecules, but not so much for neutral compounds. (Taylor. 2012).

2.3.4 Quadrupole

Quadrupoles are filters which allow components to pass through them based on their *m/Z* ratios. They are widely used in combined mass spectrometry systems such as the LC-QTOF-MS. (Ketola, Kostiainen, Kotiaho & Vainiotalo. 2010.) The quadrupole mass analyser consists of four parallel rods spaced equidistantly, the opposite pairs electrically connected (Comsol Inc. 2018.)

After the quadrupole the ions are directed into the collision cell where they are accelerated into an inert gas such as nitrogen or argon and each ion collides with gas molecules at different collision energies. This causes the precursor ions to break into fragment ions which are then directed into the time-of-flight analyser. (Ketola, Kostiainen, Kotiaho & Vainiotalo. 2010.)

2.3.5 Time-of-flight Analyzer

In the time-of-flight mass analyser, the beam of ions first passes into the ion pulser. Using high voltage, the ions are accelerated in direction parallel to their original path in the flight tube which is about one-meter long. At the end of the flight tube there is an electrostatic ion mirror which reverses the direction of the ions. Because ions enter the ion pulser with some horizontal momentum, they are not reflected back to the ion pulser, but towards the detector as demonstrated in Figure 1. (Agilent Technologies Inc. 2011.)

2.4 Centrifugal vacuum concentrator

Centrifugal vacuum concentrator is a combination of centrifugation, vacuum and heat to concentrate the samples until completely dry. The instrument has adjustable heating levels and is ideal for concentrating aqueous and organic solvents. The system is equipped with a cold trap, which protects the vacuum pump from the corrosive chemicals as they evaporate from the samples. (Labconco. 2018.)

3 AIM OF STUDY

The samples sent to the Forensic Toxicology Service were previously analyzed using separate methods for different drugs and different sample preparation suitable for each sample type. Some drugs, however, have very similar retention times and therefore could not be properly separated using the previous methods and instrumentation. In cases like this the liquid chromatography (LC) quadrupole time-of-flight (QTOF) mass spectrometer (MS), later referred to as LC-QTOF-MS, enabled separating and analyzing these compounds faster and more accurately. The previous methods were time-consuming, and there was a shortage of personnel as well, which is why ASI were having difficulties delivering results within the given turnaround times, especially for complex cases. Variation in the samples and substances of interest resulted in necessity to perform the assays with different methods and instrumentation. The aim of this thesis was to test a new method for LC-QTOF-MS for qualitative and quantitative analysis in post-mortem samples as a part of the method development procedure. The objective was to make it possible to get accurate results to report within the given turnaround times by reducing the number unnecessary assays.

The typical turnaround time is fifteen days from the arrival of the samples to the reporting of results, but it varies with some clients. This new method was required to enable screening of blood and urine samples for all the drugs and metabolites in them and quantify many of the most common ones found in post-mortem samples simultaneously without requiring further analysis. It would significantly reduce the number of separate assays carried out on different instruments and shorten the time required to obtain results for each case making it possible to deliver results within the given turnaround times. It would also make it possible to screen samples for newer, recreationally used drugs previously not tested for unless specifically requested.

Another objective was to reduce the number of active cases at a time. ASI receives anywhere between 1-6 samples per case, sometimes more. When a case is reported and finished the samples are frozen down and stored for a minimum of 6 months, but in some cases the samples may be retained for even up to 30 years.

4 MATERIALS AND METHODS

4.1 Instrumentation

The method was developed and tested using an Agilent 1290 II liquid chromatography system equipped with a 6545 QTOF-MS. The Agilent MassHunter All Ions MS/MS software was used for qualitative and quantitative analysis on the instrument. (Josefsson, M. & Roman, M. 2013.) The compounds found in samples were identified by comparing their *m/Z* ratios and retention times with the MS/MS spectral libraries and the chromatographic coelution of the precursor and fragment ions. (Agilent Technologies Inc. 2014.)

4.2 Reagents

All quality control samples, calibrators and internal standards for post-mortem blood samples were prepared using the Blood QTOF Quantification Workflow proposed by Couchman and Hecht (2018), based on commercially available drug and metabolite sub-mixes by Agilent Technologies. Each of the mixes were supplied as 100 mg/L solutions and were used to prepare a combined 1 mg/L stock solution.

The drugs and metabolites included in the calibrator solutions could also be quantified simultaneously with the basic LC-QTOF-MS drug screen. Other compounds in the specimens, but not in the list of compounds included in the sub-mixes, were identified based on the data in the spectral library and quantified separately with another method if necessary.

4.2.1 Blank blood and urine

The blank specimens of blood and urine used for the analysis were from blood and urine donors previously screened and confirmed to contain no drugs. The blank specimens were stored in freezers and fridges carefully labelled with their own case and sample numbers.

4.2.2 The Agilent LC/MS forensic toxicology standard sub-mixes

Sub-mix 2

This sub-mix consists of stimulant drugs (2.2.16) and their metabolites. Stimulant drugs and metabolites included in the sub-mix 2 are listed in Table 1.

TABLE 1. Stimulant drugs included in sub-mix 2. (Couchman & Hecht. 2018.)

Stimulant Drugs	
Atropine	Lidocaine
Benzoylecgonine	Methylenedeoxyamphetamine (MDA)
Caffeine	3,4- methylenedeoxymethamphetamine or ecstasy (MDMA)
Carisoprodol	Methylenedioxy-N-ethylamphetamine (MDEA)
Cocaethylene	Methamphetamine
Cocaine	Methylphenidate
Cotinine	Nicotine
Amphetamine	Phencyclidine (PCP or Angel Dust)
Dextromethorphan	Phentermine

Sub-mix 3

Benzodiazepines, nonbenzodiazepines and benzodiazepine receptor antagonists (2.2.9.) are included in the sub-mix 3. Benzodiazepines are listed in Table 2, nonbenzodiazepines and benzodiazepine receptor antagonists in Table 3.

TABLE 2. Benzodiazepines included in the sub-mix 3. (Couchman & Hecht. 2018.)

Benzodiazepines	
7-Aminoclonazepam,	Diphenhydramine
7-Aminoflunitrazepam	Fenazepam
Alpha-hydroxyalprazolam	Flurazepam
Alpha-hydroxymidazolam	Lorazepam
Alpha-hydroxytriazolam,	Medazepam
Alprazolam,	Nitrazepam
Bromazepam	Nordiazepam
Chlordiazepoxide	Oxazepam
Clobazam	Prazepam
Clorazepate	Temazepam
Desalkylflurazepam	Triazolam
Diazepam	

TABLE 3. Nonbenzodiazepines and benzodiazepine receptor antagonists included in the sub-mix 3. (Couchman & Hecht. 2018.)

Nonbenzodiazepines
Zolpidem
Zopiclone
Benzodiazepine Receptor Antagonists
Flumazenil

Sub-mix 4

The sub-mix 4 consists of typical (first-generation) and atypical (second-generation) antipsychotic drugs (2.2.7). Typical antipsychotics included in the sub-mix 4 are presented in table 4 and atypical antipsychotic drugs in table 5.

TABLE 4. Typical antipsychotic drugs included in sub-mix 4. (Couchman & Hecht. 2018.)

First-generation antipsychotics	
Amisulpride	Pipamperone
Chlorprothixene	Promazine
Flupenthixol	Promethazine
Haloperidol	

TABLE 5. Atypical antipsychotic drugs and metabolites included in sub-mix 4. (Couchman & Hecht. 2018.)

Second-generation antipsychotics	
Clozapine	Risperidone
Norclozapine	Quetiapine
Olanzapine	Paliperidone

Sub-mix 6

The sub-mix 6 consists of antidepressant drugs (2.2.3). Cyclic antidepressants are listed in Tables 6 and 7. SSRIs and SNRIs are presented in Table 8.

TABLE 6. Tricyclic antidepressants included in the sub-mix 6. (Couchman & Hecht. 2018.)

Tricyclic antidepressants	
Amitriptyline	Imipramine
Amoxapine	Nortriptyline
Clomipramine	Opipramol
Desipramine	Protriptyline
Dothiepin	Trimipramine
Doxepin	

TABLE 7. Tetracyclic antidepressants included in the sub-mix 6. (Couchman & Hecht. 2018.)

Tetracyclic antidepressants
Maprotiline
Mianserin
Mirtazapine
Trazodone

TABLE 8. SSRIs and SNRIs included in the sub-mix 6. (Couchman & Hecht. 2018.)

Selective serotonin reuptake inhibitors
Citalopram
Fluoxetine
Fluvoxamine
Sertraline
Paroxetine
Serotonin-norephedrine reuptake inhibitors
Venlafaxine

Sub-mixes 9 A, B, C, D and Pholcodine

These sub-mixes consist of the narcotic analgesics (2.2.13) included in the full toxicology analysis. Opioid drugs included in the sub-mixes are represented in Table 9. Pholcodine was not originally included but was found to be useful. Therefore, pholcodine was added separately into the final mix.

TABLE 9. Opioid drugs included in the sub-mixes 9 A, B, C, D and Pholcodine. (Couchman & Hecht. 2018.)

Opioid drugs	
6-MAM	Morphine glucuronide
Acetylcodeine	Norbuprenorphine
Alfentanil	Norfentanyl
Buprenorphine	Normeperidine
Codeine	Norpropoxyphene
Dihydrocodeine	Desmetramadol
Fentanyl	Oxycodone
Hydrocodone	Oxymorphone
Hydromorphone	Pentazocine
Meperidine	Pholcodine
Morphine	Propoxyphene
2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine (EDDP)	Tramadol

4.2.3 Calibration standards

The 1 mg/L calibrator stock solution was prepared using the Agilent LC/MS forensic toxicology standard sub-mixes 2, 3, 4, 6, 9A, 9B, 9C, 9D and Pholcodine as shown in Table 10. By comparing the sample peaks with the calibration line concentrations on the Agilent MassHunter Quantitative Analysis software it was possible to quantify drugs in the samples.

All of the calibrator standards were prepared diluting the 1mg/L stock solution stock down to lower concentrations, the lowest of which being 0.005 mg/L. The dilution chart is shown in Table 15.

TABLE 10. The Calibrator 1, or the Stock Solution of 1 mg/L concentration is made by adding 50 µL of the sub-mixes 2, 3, 4, 6, 9A, 9B, 9C and Pholcodine. The concentration of sub-mix 9D is only 50 mg/L, which is the reason 100 µL of 9D is added to the Stock Solution. (Couchman & Hecht. 2018.)

Calibrator Stock Solution 1 mg / L			
Sub-mix	**Concentration of Submix**	**Volume of stock to be added**	**Volume of 50% v/v Methanol**
Sub-mix 2	100 mg/L	50 µL	4500µL
Sub-mix 3		50 µL	
Sub-mix 4		50 µL	
Sub-mix 6		50 µL	
Sub-mix 9A		50 µL	
Sub-mix 9B		50 µL	
Sub-mix 9C		50 µL	
Pholcodine		50 µL	
Sub-mix 9D	50 mg/L	100 µL	

4.2.4 Internal standard solution

The internal standard was prepared using Agilent certified solution standards which each included one of the drugs listed in Table 11. In the internal standards the drugs had been modified with deuterium (D), which is a stable isotope of hydrogen. Comparing to protium, the most common isotope of hydrogen, deuterium has an extra neutron, which makes it heavier (A. Helmentstine. 2017.) The molar mass of deuterium is 2,014 g/mol.

The deuterated drug has the same retention time and chemical formula as the unmodified drug but is slightly heavier. The LC-QTOF-MS recognized the deuterated drugs as the internal standard based on their accurate mass (e.g. the molecular mass of amphetamine is 135, 21 g/mol but the weight of the deuterated equivalent, amphetamine-D11, is 146,27 g/mol).

TABLE 11. The Internal Standard solution was made to contain 0,1 mg/L of all the deuterium modified drugs listed below. The concentration of stock solutions for each of these analytes was 1 mg/L. (Couchman & Hecht. 2018.)

Internal Standard Solution	
Amphetamine-D11	Dihydrocodeine-D6
Diazepam-D5	Morphine-D3
Haloperidol-D4	Amitriptyline-D3
Fluoxetine-D6	Methadone-D9
Benzoylecgonine-D8	Oxycodone-D6
MDMA-D5	Alprazolam-D5

4.2.5 Quality control samples

One of each quality control samples of 0.25 and 0.025 mg/L concentrations were required for each analysis. The 0.25mg/L‘high QC’ was prepared by adding each of the sub-mixes 2, 3, 4, 6, 9A, 9B, 9C, 9D and pholcodine to a 20mL volumetric flask which was then made to volume with 50% v/v methanol as shown in Table. The newly prepared 0.25mg/L solution was then diluted 1:10 in another flask for the 0.025mg/L quality control sample, also referred to as the ‘low QC’.

TABLE 12. The 0.25mg/L quality control sample, also referred to as the 'high QC' was prepared adding sub-mixes 2, 3, 4, 6, 9A-D and pholcodine into a 20mL volumetric flask and making into volume with 50% v/v methanol. (Couchman & Hecht. 2018.)

'High' Quality Control (0.25 mg/L)			
Sub-mix	**Concentration of Submix**	**Volume of stock to be added**	**Volume of 50% v/v Methanol**
Sub-mix 2	100 mg/L	50 µL	19,5 mL
Sub-mix 3		50 µL	
Sub-mix 4		50 µL	
Sub-mix 6		50 µL	
Sub-mix 9A		50 µL	
Sub-mix 9B		50 µL	
Sub-mix 9C		50 µL	
Pholcodine		50 µL	
Sub-mix 9D	50 mg/L	100 µL	

4.3 Mobile phases

The mobile phase solutions for the LC-QTOF-MS were prepared using a special QTOF-grade water and HPLC grade methanol to avoid blockage in the instrument. The formic acid used was of 99.8% purity. The two mobile phases, A and B, were prepared as shown in Table 13.

TABLE 13. Preparation of LC-QTOF-MS mobile phases A and B. (Couchman & Hecht. 2018.)

Mobile phase A	**Mobile phase B**
1000 mL of water	1000 mL of methanol
100 µL of 99.8% Formic acid	100 µL of 99.8% Formic acid
0.315g of Ammonium formate	0.315 g of Ammonium formate

4.4 Worklist

The worklist was used as a base throughout the analysis. All dilution schemes, identification numbers for blank matrices and other notes were recorded on it for later use. The worksheet included a list of samples to be analyzed with case numbers and names of the subjects. An example of a worklist is presented in Appendices 3 and 4.

On the worksheet there was a list of checks to be signed. When a sample was transferred from a container to another a check was needed to avoid mistakes. The sample check was carried out by a colleague by comparing the sample number on the original container to the labelling on the new container to make sure they match. Once the labels were checked, the worklist form was signed.

4.5 Sample preparation

For optimal results with QTOF LC/MS the post-mortem blood samples had to be prepared the right way to remove all interfering components without ruining or removing any of the analytes in them. The sample preparation procedure for post-mortem blood samples consisted of making the required dilutions of the calibrator stock solution, sample preparation, evaporation of samples to dryness and reconstitution.

Calibrator dilutions were prepared by diluting the 1 mg/L calibrator stock solution into five calibrators of the following concentrations: 0.5 mg/L, 0.1 mg/L, 0.05 mg/L, 0.01 mg/L and 0,005 mg/L. The instructions for the calibration dilutions can be found in Table 15. A blank sample (0 mg/L) was placed after the calibrators and consisted only of blank blood and 50% methanol.

Two quality control samples were added to each batch, one of low (0,025 mg/L) and one of high (0,25 mg/L) concentration. The lower concentration one came after the blank, and the higher one was placed at the end of the batch. An example of a typical batch of samples is presented in Appendix 3.

For sample preparation 100µL of each sample, calibration standard, quality control was pipetted into 2 mL Eppendorf tubes. All samples needed to be in the same matrix with

the calibration standards and quality controls. As the calibrators and quality controls were prepared using 50% v/v methanol and the samples were blood, serum or vitreous humour, to make them all into same matrix, 100µL of blank blood was added to the calibrators standards and controls, and 100µL of 50% methanol was added to each of the samples. 10µL of internal standard solution was added to each of the samples, standards and quality controls. The sample preparation and matching of the matrices is presented in Table 15.

When all samples, calibrators standards and quality controls were in the same matrix, some acetonitrile was added to coagulate the proteins, blood cells and other interfering components. Originally 1 mL of acetonitrile was used for this step, but it was later reduced to 750µL, as it worked equally well, but dried up significantly faster in the vacuum concentrator. With appropriate mixing and then a spin in the microfuge all interfering components were nicely pressed to the bottom of the tubes while the analytes remained in the supernatant.

TABLE 14. The stock solution of 1 mg/L concentration and is used as it is for Calibrator 1. For the Calibrators 2-4 the Stock Solution is diluted with 50% QTOF grade Methanol according to table. Calibrators 5 and 6 are diluted using the previous Calibrators 3 and 4. (Couchman & Hecht. 2018.)

Calibration standard	**Target Concentration**	**Stock Solution to be added**	**50% MeOH to be added**
Cal 1 (Stock solution)	1 mg/L	Ready as it is	0 µL
Cal 2	0.5 mg/L	250 µL	250 µL
Cal 3	0.1 mg/L	50 µL	450 µL
Cal 4	0.05 mg/L	25 µL	475 µL
Cal 5	0.01 mg/L	100 µL of Cal 3	900 µL
Cal 6	0.005 mg/L	100 µL of Cal 4	900 µL

TABLE 15. Sample preparation and matching of the matrices for all samples, calibration standards and quality controls. (Couchman & Hecht. 2018.)

Calibration standards	Quality controls	Samples
100µL of standard diluted to target concentration	100µL of quality control sample (High or Low)	100µL of sample blood, vitreous humour or serum
100µL of blank blood	100µL of blank blood	100µL of 50% methanol
10µL of internal standard	10µL of internal standard	10µL internal standard
750µL of acetonitrile	750µL of acetonitrile	750µL of acetonitrile

4.6 Evaporation

After centrifugation the supernatants were decanted into labeled 4.5 mL polypropylene tubes leaving the interfering components behind. The polypropylene tubes were placed in the SpeedVac vacuum concentrator to spin and dry out for approximately 2,5 hours.

4.7 Reconstitution

When the samples had completely dried in the SpeedVac they were reconstituted into 200µL of 20% v/v Methanol in QTOF grade water. The reconstituted samples were then transferred into reduced volume autosampler vials and firmly capped to avoid evaporation of the contents.

4.8 Setup and tuning of LC-QTOF-MS

The instrument was calibrated weekly and an Auto Tune was performed every day before the first sample run to calibrate the instrument. Agilent MassHunter Data Acquisition software was used for both qualitative and quantitative analysis on the LC-QTOF-MS instrument in its All Ions data acquisition mode.

5 RESULTS

The instrument was set up to compare all analytes' retention times and exact masses to the data in the library using the MassHunter Quantitative Analysis software. The software showed a list of analytes that were qualified, meaning that the analyte in question was detected in the sample. The software also created a calibration line using the measured concentrations from the calibration standards as shown in Figure 2.

Each QTOF blood or urine analysis batch was checked by comparing the masses, retention times and fragments of each precursor ion. Each batch was checked twice, once by a toxicologist and then by the study director to confirm. There were, however, some cases where the result was not as obvious as it seemed at first, which is why the results suggested by the software needed to be checked by a toxicologist. The instrument and the software alone could not make certain conclusions.

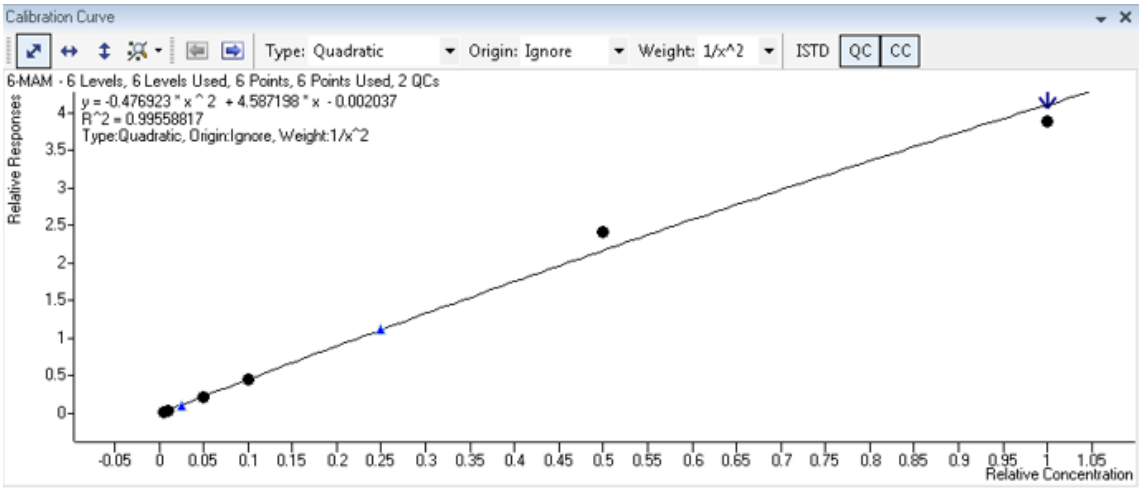

Figure 2. Calibration line created by the MassHunter Quantitative Analysis software based on the measured concentrations of the calibration standards.

5.1 Positive result

When a detected analyte matched the data in the library in exact mass and retention time and showed fragments, it was considered a positive result. Figures 3 and 4 demonstrate clearly positive results for cocaine and its metabolite benzoylecgonine.

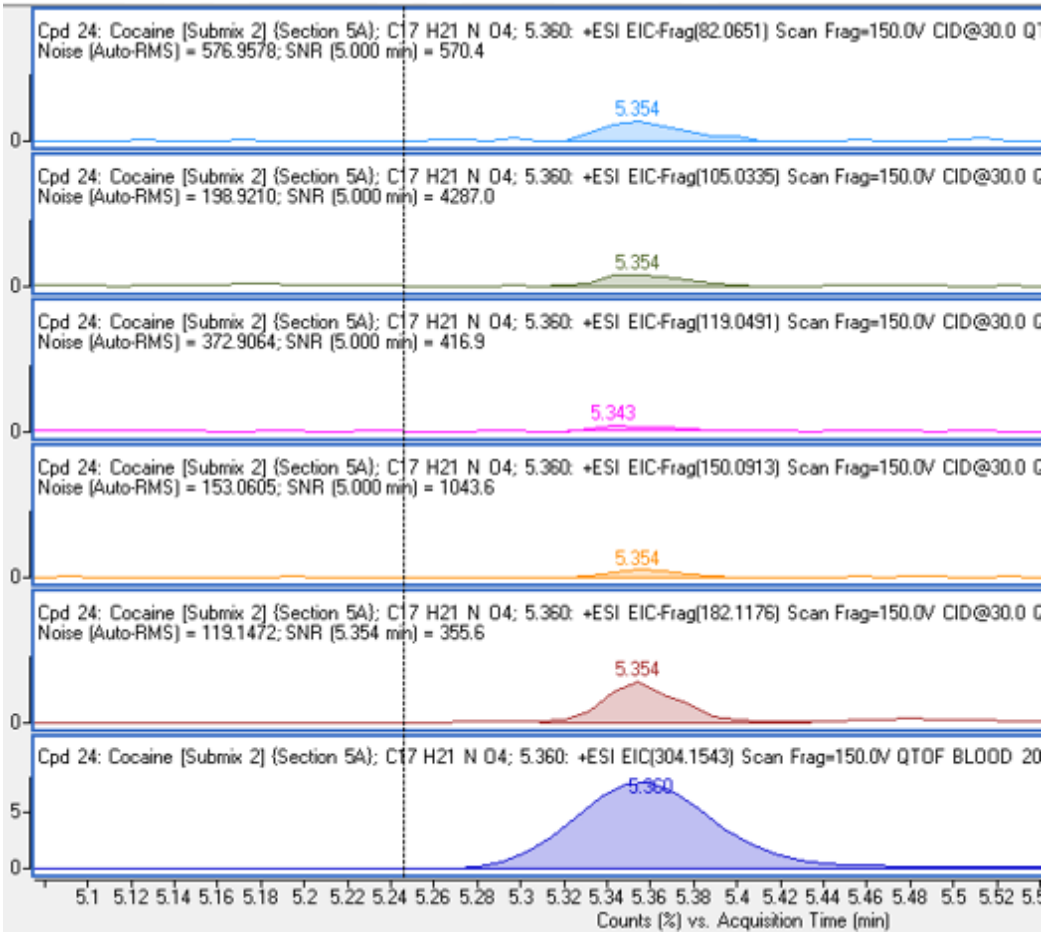

Figure 3. Positive result for cocaine. The fragment in the bottom in dark blue is the precursor ion and the five peaks above it are its fragment ions. The retention times on each fragment are within acceptable limits.

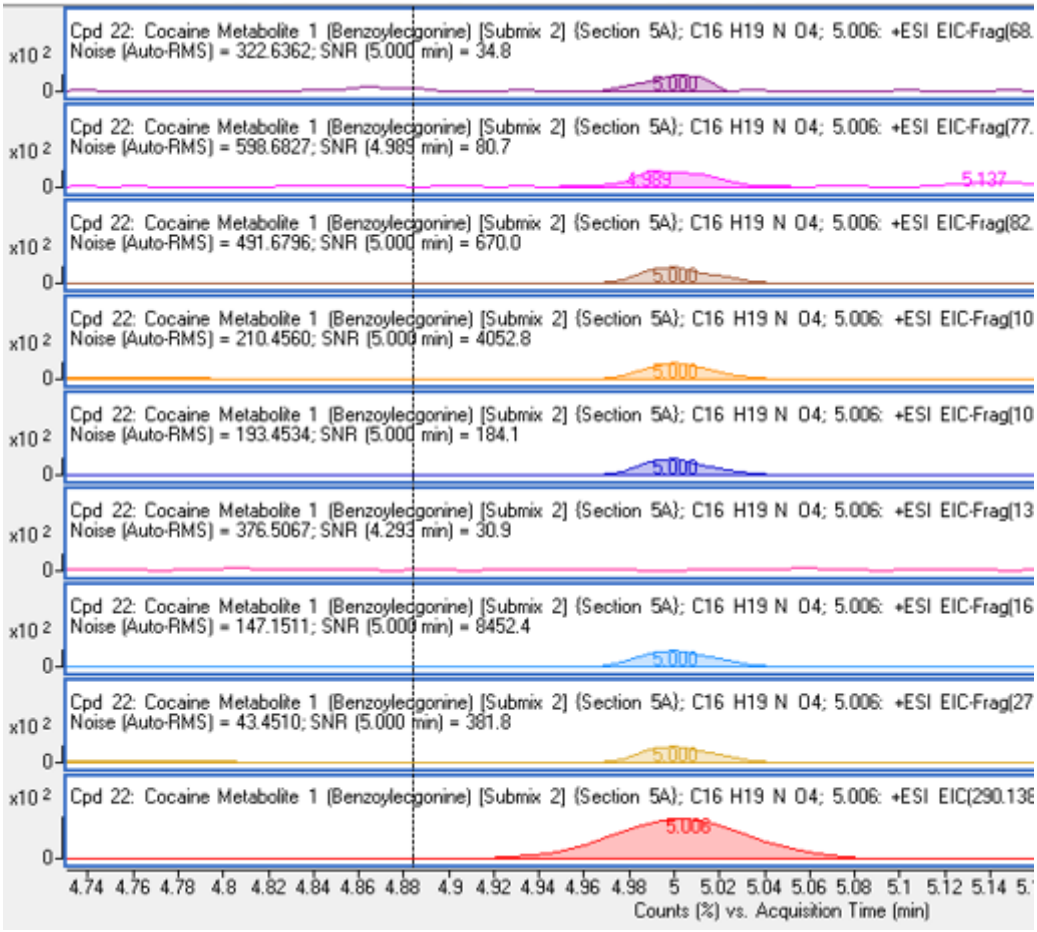

Figure 4. Clear positive result for the main metabolite of cocaine, benzoylecgonine. The precursor ion is shown in the bottom in red, the other ones above are its fragment ions.

5.2 Negative result

In figure 5 a clearly negative result is presented for pentylone, a stimulant drug developed in the 1960s. In the figure it shows the precursor ion, but no fragment ions, and therefore the result is negative.

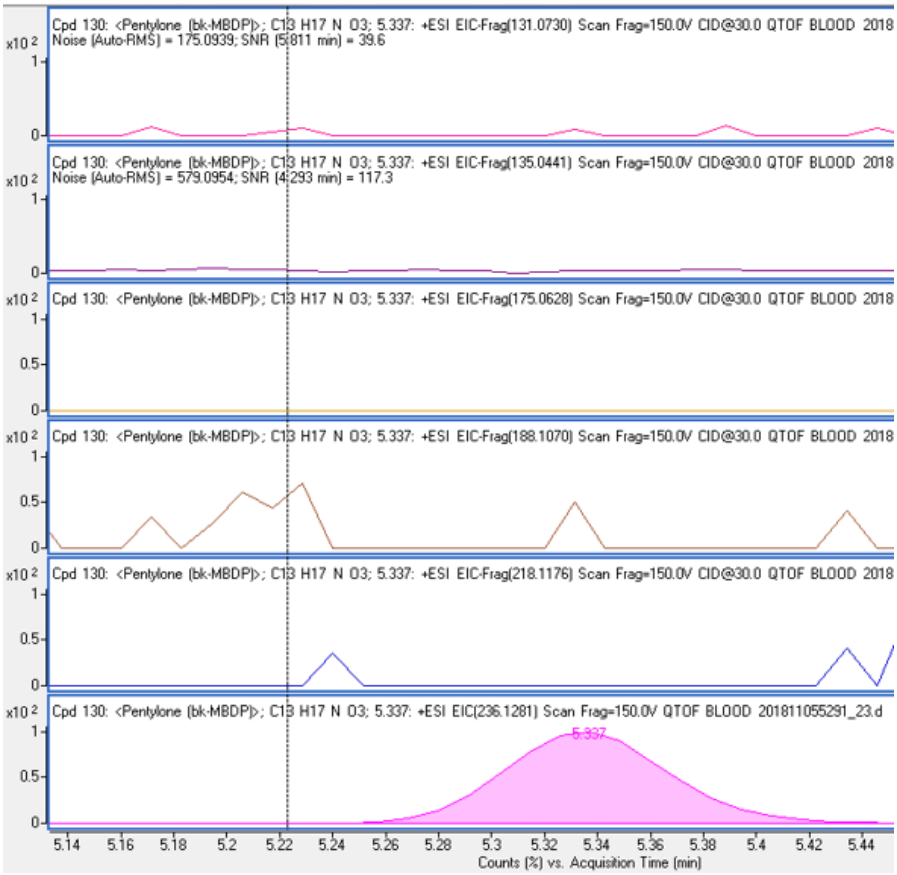

Figure 5. Negative result for pentylone.

5.3 More complicated results

5.3.1 Saturated detector

Figure 6 presents a result for pregabalin. By the looks of it, it is a negative result as the fragment ion peaks are larger than that of the precursor ion, which is not possible. When looking at its two isotopes pregabalin M+1 (Figure 7) and pregabalin M+2 (Figure 8) the result is clearly positive. The concentration of pregabalin in this case was so high, that it saturated the detector and the masses were no longer in the 20ppm mass window.

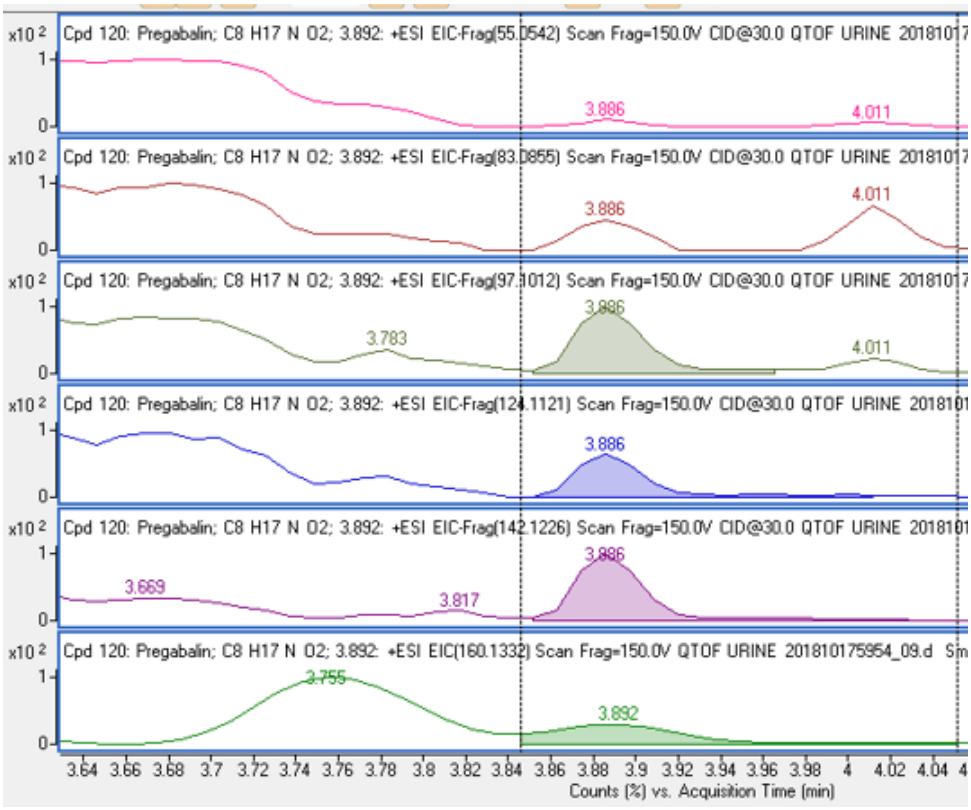

Figure 6. Pregabalin precursor ion in the bottom in green, and its supposed fragment ions above it. Fragment ions are larger than that of their parent ion, which makes it appear a negative result.

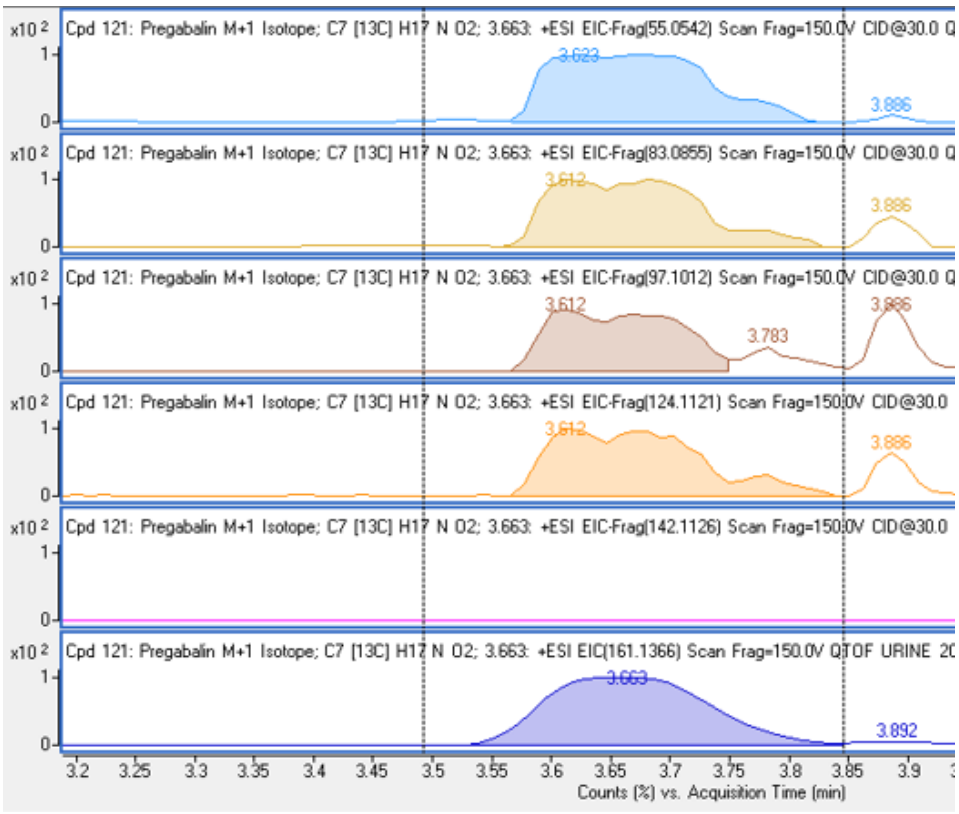

Figure 7. Pregabalin isotope M+1 shows the precursor ion in dark blue and its fragment ions above. The result is positive.

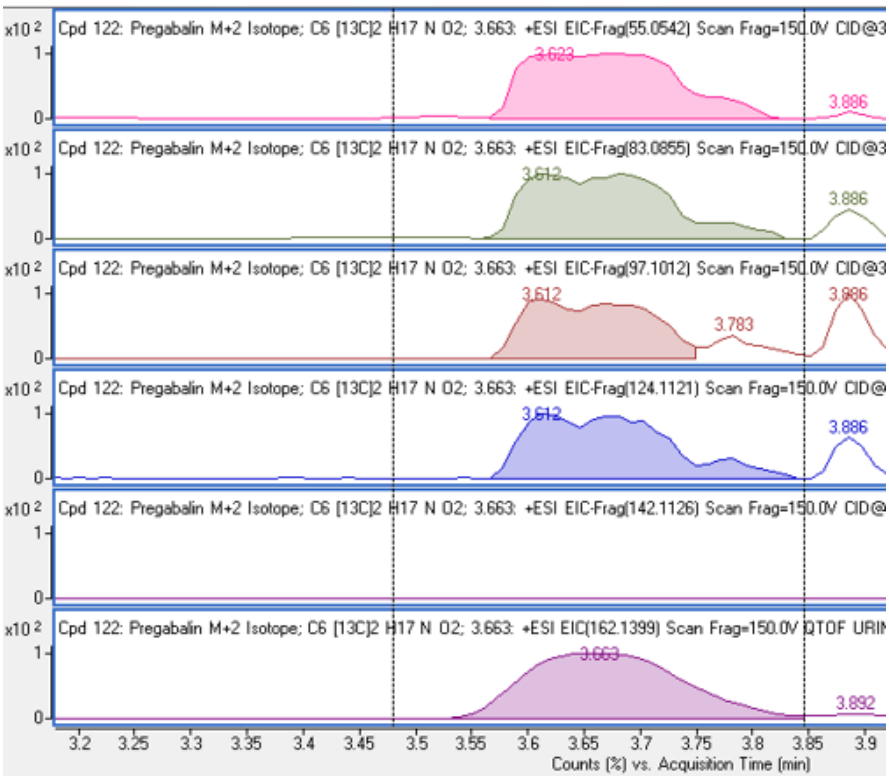

Figure 8. Pregabalin isotope M+2 shows precursor ion in purple and its fragment ions above it. The result is positive.

5.3.2 Compounds with same accurate mass

Some drugs and metabolites have the same mass but different retention times and vice versa. Figure 9 presents a positive result for galantamine but the retention time of 3.685 does not match that of galantamine (3.19 minutes). Nordihydrocodeine has the same accurate mass as galantamine, and its retention time would be 3.69 minutes. In this case the software misidentified nordihydrocodeine as galantamine and the result was determined by the difference in retention times.

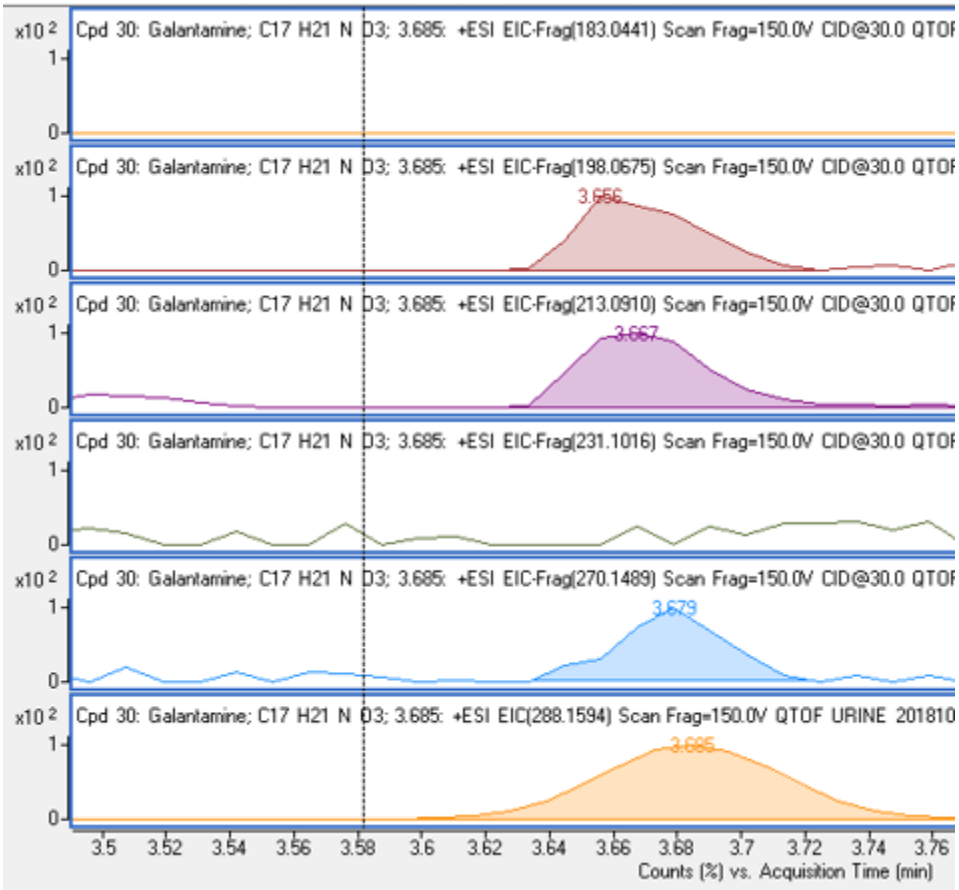

Figure 9. Nordihydrocodeine was misidentified as galantamine because of the two having the same accurate mass. The retention time determines that this compound in question is nordihydrocodeine.

6 DISCUSSION

The aim of this thesis was to hasten the full toxicology analysis on blood samples by testing a new method developed for post-mortem blood samples on the LC-QTOF-MS. Introducing the combined screening and quantification on LC-QTOF-MS improved the turnaround times significantly by reducing the number of unnecessary assays.

On the new instrument dozens of samples can be analyzed in one batch, only the sample preparation is time-consuming when it comes to blood samples. The instrument analyzes the samples in a relatively short time, 12 minutes for screening and quantification per sample. Previously, for a full toxicology drug screen a minimum of nine assays were run. Any prescribed drugs not included in those nine assays were added additional, which is why the full toxicology screen often required somewhere between 10 and 15 separate assays or even more. The old way was to screen the blood specimens for basic drugs, benzodiazepines and salicylate separately using gas chromatography-mass spectrometer (GCMS). An estimated concentration was obtained from any positive samples using a single point calibrator. If the estimated concentration was above therapeutic range, it was accurately quantified accompanied by a full calibration line and quality controls. Any other drugs detected on GCMS that were not included in the calibrator mix were quantified as no estimate concentration was available. Each of the separate assays for screening and quantification of drugs took 5-30 minutes per sample. With LC-QTOF-MS the number of assays on specimens where no drugs were detected went from a minimum of nine assays down to four – the QTOF assays for blood and urine and the GCMS assays for cannabinoids and ethanol. Previously even clean samples would have required at least 13 assays plus any additional assays for drugs deceased may have been prescribed but not taking.

In the Journal of Analytical Toxicology (2013; 37(1): 17–24) a similar study by Guale et al. using a liquid chromatography time-of-flight mass spectrometry system (LC-TOF-MS). The aim of the study was to validate a method for screening for drugs, metabolites and collateral compounds in forensic toxicology specimens. Despite the equipment and parameters being slightly different the validated method resulted in rapid, enhanced screening for blood and urine specimens in forensic toxicology casework and agrees with the results of this thesis.

A year later another similar study by Rosano, Na, Ihenetu, Swift and Wood (Journal of Analytical Toxicology. 2014; 38: 495–506) was carried out using an ultra-performance liquid chromatography time-of-flight mass spectrometry system (UPLC-MS/TOF). The aim of the study was to develop a method for UPLC-MS/TOF for screening and simultaneous quantification of sixteen drugs and metabolites frequently confirmed in post-mortem blood samples. In both studies it was clear that multi-analyte quantification improves workflow in the laboratory and results in improved turnaround times by reducing the number of assays, as it did in this thesis. Therefore the results of this thesis can be considered accurate and reliable. LC-QTOF-MS does indeed improve the full toxicology analysis in forensic toxicology.

Possible ideas for further development would be to shorten the sample preparation time by further decreasing the amount of acetonitrile used in the precipitation step. The volume of acetonitrile used was decreased before from 1000µL into 750µL which shortened the time it took for the samples to dry out in the vacuum concentrator. Maybe decreasing it to 500µL would shorten it further. The acetonitrile was used to remove proteins from blood specimens, so decreasing the amount of it may not remove the proteins in the specimens efficiently enough. Another development idea for shortening the sample preparation time would be to use better quality samples. In this case the quality of the samples is not something ASI can really affect. Asking the clients to provide a preserved blood sample if possible would at least make the results more reliable when no post-mortem redistribution affects the drug concentrations in the samples.

Also checking results after the run is quite slow and the drugs detected are recorded on a paper form by hand. Bringing the qualified samples up on a sheet on the computer where the toxicologist could simply make a note next to each drug and metabolite detected whether they are there or not could be useful. It would save a lot of writing and paper.

Blood and urine samples are analyzed in separate batches because of the more complex sample preparation procedure necessary for blood samples. Urine samples are ready for analysis with a simple dilution. Samples of vitreous humour and serum are prepared the same way as blood, so they can be analyzed together with blood samples. Sections of tissue, which are submitted for analysis in the absence of blood, have their own sample preparation procedure and are therefore analyzed separately from the other specimens. Currently, the analysis of tissue specimens gets postponed until there are enough samples,

at least 15 or so, for a batch to run on LC-QTOF-MS as analyzing smaller batches is not very efficient. If there was a way to prepare and analyze samples of blood, serum, vitreous humour and sections of tissue efficiently in the same batch it would enable analysis of more samples in a shorter time.

7 SOURCES

Amboss. 2018. https://www.amboss.com/us/knowledge/Antiarrhythmic_drugs. Cited on December 7, 2018.

Agilent Technologies Inc. 2011. Time-of-flight Mass Spectrometry. http://hpst.cz/sites/default/files/attachments/5990-9207en-time-flight-mass-spectrometry.pdf.

Agilent Technologies, Inc. 2014. Concepts guide. Agilent 6200 Series TOF and 6500 Series QTOF LC/MS System.

Agilent Technologies, Inc. 2017. Revision A. January 2017. Agilent 6200 Series TOF and 6500 Series Q-TOF LC/MS System. Quick Start Guide.

Alzheimer's Society. United Kingdom. Reviewed 2018. Antipsychotic Drugs. https://www.alzheimers.org.uk/about-dementia/treatments/drugs/antipsychotic-drugs. Cited on December 4th, 2018.

American Diabetes Association. Updated 2016. DKA (Ketoacidosis) & Ketones, Hyperosmolar Hyperglycemic Nonketotic Syndrome (HHNS). http://www.diabetes.org/living-with-diabetes/complications/ketoacidosis-dka.html. Cited on December 8, 2018.

Baumann, M. H. 2014. Awash in a sea of 'bath salts': implications for biomedical research and public health. Addict (Abingdon England). 2014;109(10):1577-1579.

Baumann, M., Partilla, S., Lehner, K., Thorndike, E., Hoffman, A., Holy, M., Rothman, R., Goldberg, S., Lupica, C., Sitte, H., Brandt, S., Tella, S., Cozzi, N & Schindler, C. 2013. Neuropsychopharmacology. Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive 'bath salts' products. 2013; 38(4): 552-62.

ChemicalSafetyFacts.org. Updated 2018. https://www.chemicalsafetyfacts.org/ethanol/. Cited on December 8, 2018.

Comsol Inc. 2018. https://www.comsol.com/model/quadrupole-mass-filter-12039. Cited on December 2, 2018.

Couchman, L & Hecht, M. 2018. Blood QTOF Quantitation Workflow.

Drugs.com. Updated 2018. https://www.drugs.com/. Cited on December 8, 2018.

Encyclopedia Britannica. 1998. Updated 2018. https://www.britannica.com/science/opium#accordion-article-history. Cited on December 4th, 2018.

Epilepsy Society. 2018. https://www.epilepsysociety.org.uk/list-anti-epileptic-drugs#.XAv9gNsza70. Cited on December 8, 2018.

GOV. UK. https://www.gov.uk/government/news/headshops-closed-and-offenders-arrested-after-legal-highs-ban. Cited on December 5, 2018.

Grotenhermen F, Müller-Vahl K: The therapeutic potential of cannabis and cannabinoids. Dtsch Arztebl Int 2012; 109(29–30): 495–501.

Guale, F., Shahreza, S., Walterscheid, J. P., Chen, H.-H., Arndt, C., Kelly, A. T. & Mozayani. A. 2013. Journal of Analytical Toxicology. Validation of LC-QTOF-MS Screening for Drugs, Metabolites, and Collateral Compounds in Forensic Toxicology Specimens. Jan; 37(1): 17–24.

Gunja, N. 2013. American College of Medical Toxicology. The Clinical and Forensic Toxicology of Z-Drugs. 9(2): 155–162. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3657020/.

Healthline Media. Reviewed 2018. https://www.healthline.com/health/depression/serotonin-norepinephrine-reuptake-inhibitors-snris. Cited on December 3, 2018.

Helmentstine, A. September 2nd 2017. ThoughtCo. Deuterium Facts – What is deuterium? Website. https://www.thoughtco.com/facts-about-deuterium-607910.

Herchline, T. E. 2018. WebMD LLC. https://emedicine.medscape.com/article/221134-medication

Houck, M. & Siegel, J. 2015. Fundamentals of Forensic Science. 3rd Edition.

Josefsson, M. & Roman, M. Agilent Technologies, Inc. 2013. USA. Josefsson, M., Roman, M., Wüst ,B., Madden, S., Payne, T. & Taylor, L. Rapid and Accurate Forensics Analysis using High Resolution All Ions MS/MS. https://www.agilent.com/cs/library/applications/5991-2319EN.pdf.

Ketola, R., Kostiainen, R., Kotiaho, T. & Vainiotalo, P. 2010. Suomen Massaspektrometrian Seura. Massaspektrometrian perusteet.)

King, L. A. & Kicman, A. T. 2011. Drug Testing Analysis 3: 401-403. A brief history of ‘new psychoactive substances.

Kratzsch, C., Peters, F. T., Kraemer, T., Weber, A. A. & Maurer, H. H. Research article. Journal of Mass Spectrometry, 2003. Screening, library-assisted identification and validated quantification of fifteen neuroleptics and three of their metabolites in plasma by liquid chromatography/mass spectrometry with atmospheric pressure chemical ionization. https://onlinelibrary.wiley.com/doi/abs/10.1002/jms.440.

Labconco. 2018. https://www.labconco.com/category/vacuum-concentrators.

Long, M. S. Chemyx Inc. 2018. https://www.chemyx.com/support/knowledgebase/applications/basic-principles-hplc-ms-lc-ms/.

Mandal, A. Updated 2018. What are Cannabinoids? https://www.news-medical.net/health/What-are-Cannabinoids.aspx

MFMER, Mayo Foundation of Medical Education and Research. 2018.

MedicineNet, Inc. 2018. https://www.medicinenet.com/

National Institute on Drug Abuse; National Institutes of Health; U.S. Department of Health and Human Services. https://www.drugabuse.gov/publications/drugfacts/synthetic-cathinones-bath-salts#ref. Updated in February 2018.

NHS UK. Reviewed 2018. https://www.nhs.uk/. Cited on August 10th, 2018.

Olson, L. G. 2008. Australian Prescriber. 31:146–9. Hypnotic hazards: adverse effects of zolpidem and other z-drugs. https://cdn0.scrvt.com/08ab3606b0b7a8ea53fd0b40b1c44f86/b615f3cfacd56819/5e0ebc86d370/Hypnotic-hazards_31-146.pdf

Pappas, S. 2017. Massive poppy bust: Why home-grown opium is rare. LiveScience. https://www.livescience.com/59452-why-opium-is-grown-outside-us.html

Premier Biosoft. 2018. http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html/. Cited on November 14, 2018.

Rosano T. G., Na, S., Ihenetu, K., Swift, T. A. & Wood, M. 2014. Journal of Analytical Toxicology. Multi-drug and Metabolite Quantification in Postmortem Blood by Liquid Chromatography–High-Resolution Mass Spectrometry: Comparison with Nominal Mass Technology. 38:495–506.

Rang, H. P., Dale, M. M., Ritter, J. M., Moore, P. K.. 2003. Elsevier Churchill Livingstone. United Kingdom. Rang and Dale's Pharmacology. Fifth Edition.

Stargate International. 2008. Party pills: successful safety record. Press release.

Taylor, T. Feb 01, 2012. LCGC North America. Electrospray Ionization for Mass Spectrometry. Vol 30, Issue 20, 182.

Thermo Fisher Scientific Inc. Updated 2016. https://www.thermofisher.com. Cited on December 8, 2018.

The Royal Children's Hospital Melbourne. https://www.rch.org.au/rch/about/terms-and-conditions/. Read on December 7th, 2018.

Thomas, S., Villa, L., Crane, M., Watkins, M., Wagener, D. & Brande, L. 2018. Recovery Brands, LLC. American Addiction Centers, Inc. https://DrugAbuse.com.

UK Addiction Treatment Centres. https://www.ukat.co.uk/opiates/. Cited on October 18, 2018

United Nations Office on Drugs and Crime. 2018. https://www.unodc.org/LSS/Page/NPS/Resources

Virtual Medical Centre. Updated 2017. https://www.myvmc.com/treatments/simple-analgesics-non-opioid-analgesics/. Cited on December 8, 2018.

von Euler, M., Villén, T., Svensson, J. O., & Ståhler, L.. 2003. Sweden. Karolinska Institutet. Huddinge University. Hospital Division of Clinical Pharmacology. Ther Drug Monit. 2003 Oct;25(5):645-8.

8 APPENDICES

Appendix 1. A list of drugs and metabolites included in the full toxicology analysis in ASI. Page 1 of 2.

Full Toxicology Analysis	
Alcohol (ethanol)	
Amphetamines and amphetamine-like compounds	Amphetamine, methamphetamine, MDMA, MDA, MDEA, para-methoxyamphetamine (PMA), para-methoxymethamphetamine (PMMA), 4-fluoromethamphetamine, methylphenidate, ephedrine, methylenedioxypyrovalerone (MDPV), diphenylprolinol (D2PM) and 2-diphenyl pyrrolidine (Desoxy-D2PM)
Piperazines screen	BZP, TFMPP, mCPP, MBZP, DBZP and MPP
Cathinones screen	methcathinone, ethcathinone, mephedrone (4- methylmethcathinone, 4-MMC), flephedrone (4-fluoromethcathinone), 4-methylethcathinone (4-MEC), pentedrone (beta-ethylmethcathinone), methedrone, dimethylcathinone, methylone and butylone
Analgesics	
Non-opioid	Paracetamol, ibuprofen and salicylates
Narcotic analgesics	alfentanil, buprenorphine, codeine, dextropropoxyphene, dihydrocodeine, dipipanone, methadone, morphine, oxycodone, pethidine, pholcodine and tramadol
Anaesthetics	Ketamine and lidocaine
Antiarrhythmics	Flecainide and propanolol
Antidepressants	Amitriptyline, nortriptyline, citalopram, clomipramine, desipramine, dothiepin (dosulepin), fluoxetine, fluvoxamine, imipramine, mirtazapine, venlafaxine, paroxetine, sertraline, trimipramine and trazodone
Antiepileptics	Carbamazepine and lamotrigine

Appendix 2. A list of drugs and metabolites included in the full toxicology analysis in ASI. Page 2 of 2.

Antihistamines	Cyclizine, chlorphenamine, diphenhydramine and promethazine
Antimalarials	Quinine
Antimuscarinic drugs	Orphenadrine, procyclidine and trihexyphenidyl
Antipsychotics	Amisulpride, chlorpromazine, clozapine, levomepromazine, olanzapine, promazine and thioridazine
Barbiturates	
Benzodiazepines	Alprazolam, chlordiazepoxide, chlordiazepoxide lactam, clobazam, clonazepam, 7-aminoclonazepam, desmethyldiazepam, diazepam, fenazepam, flurazepam, loprazolam, lorazepam, lormetazepam, medazepam, midazolam, nimetazepam, nitrazepam, oxazepam and temazepam
Cannabinoids	
Cocaine and benzoylecgonine	
Hypnotics	Zaleplon and zolpidem
Markers of ketoacidosis	Acetone and beta-hydroxybutyrate

Appendix 3. Simplified version of typical worklist. Page 1 of 2.

Assay		QTOF Blood			
Assay date:		19 June 2018			
Method:		QTOF	Analyst name:		
Instrument:		Agilent QTOF	Analyst signature:	*Siiri Kosonen*	
1	1	Cal 1	1 mg/L	Calibrator	
2	2	Cal 2	0.5 mg/L	Calibrator	
3	3	Cal 3	0.1 mg/L	Calibrator	
4	4	Cal 4	0.05 mg/L	Calibrator	
5	5	Cal 5	0.01 mg/L	Calibrator	
6	6	Blank	0 mg/L	Calibrator	
7	QC Low	QC	0.025 mg/L	Control	
8	Case1	Sample No. 1	SURNAME, Forename	Blood (PM) Preserved	
9	Case2	Sample No. 2	SURNAME, Forename	Blood (PM)	
10	Case3	Sample No. 3	SURNAME, Forename	Blood (AM)	
11	Case4	Sample No. 4	SURNAME, Forename	Liver	
12	Case 5	Sample No. 5	SURNAME, Forename	Muscle	
13	Case 6	Sample No. 6	SURNAME, Forename	Blood (PM) Preserved	
14	Case 7	Sample No. 7	SURNAME, Forename	Blood (PM)	
15	Case 8	Sample No. 8	SURNAME, Forename	Blood (AM)	
16	Case 9	Sample No. 9	SURNAME, Forename	Blood (PM)	
17	Case 10	Sample No. 10	SURNAME, Forename	Blood (PM)	
18	Case 11	Sample No. 11	SURNAME, Forename	Blood (PM)	
19	Case 12	Sample No. 12	SURNAME, Forename	Blood (PM)	
20	QC High	QC	0.25 mg/L	Control	
Worksheet_Ref No: QTOF Blood_YYYYMMDD1234					Page 1 of 2

Appendix 4. Simplified version of typical worklist. Page 2 of 2.

Assay	QTOF Blood		
Assay date:	19 June 2018		
Method:	QTOF	Analyst name:	S. Kosonen
Instrument:	Agilent QTOF	Analyst signature:	*Siiri Kosonen*

Calibrator:	Stock reference
1 mg/L	

Pipette	Pipette number
10 – 100µL adjustable	
50 – 200µL adjustable	
50 – 250µL adjustable	
100 – 1000µL adjustable	
Multipipette	

Quality Control stock reference

Low QC 0,025 mg/L

High QC 0,25mg/L

Internal standard reference / conc. / volume used

1 mg/L

Blank matrix reference

CM_999

Calibrator / QC /

Sample check

Additional assay checks:

Check 1 *Decanting order*

Check 2 *Vial transfer*

Check 3 *Autosampler vial order*

Calibrator / Quality Control preparation, dilution scheme, general notes etc.

Calibrator	*Concentration*	*Vol of Stock*	*Vol. of MeOH*
1	*1*	*-*	*0*
2	*0.5*	*250*	*250*
3	*0.1*	*50*	*450*
4	*0.05*	*25*	*475*
5	*0.01*	*100 (3)*	*900*
6	*0.005*	*100 (4)*	*900*

SOP / Method Flowchart Reference	Deviations / method changes

Worksheet_Ref No: QTOF Blood_YYYYMMDD1234 Page 2/2