UNIVERSITY OF TARTU
FACULTY OF SCIENCE AND TECHNOLOGY
Institute of Chemistry

Rūta Veigure

DEVELOPMENT AND VALIDATION OF UHPLC-MS/MS METHOD FOR ANALYSIS OF SEDATIVE DRUGS AND THEIR METABOLITES IN BLOOD PLASMA

Master's thesis (30 EAPs)
Applied measurement science

Supervisors:
Assoc. prof. Koit Herodes
Karin Kipper, PhD

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LIST OF ABBREVIATIONS

3xLLOQ – low quality control sample (3 times lowest limit of quantification)

DLLME - dispersive liquid-liquid microextraction

ESI – electrospray ionization

HFIP – 1,1,1,3,3,3-hexafluoro-2-propanol

HPLC – high performance liquid chromatography

ICU – intensive care unit

ID – internal diameter

IS – isotope labelled internal standard

LC-MS – liquid chromatography-mass spectrometry

LLE – liquid–liquid extraction

LLOQ – lowest limit of quantification

M3G – morphine-3-β-glucuronide

M6G – morphine-6-β-glucuronide

M3G-D_3 – morphine-3-β-glucuronide-D_3

M6G-D_3 – morphine-6-β-glucuronide-D_3

MeCN – acetonitrile

MED – medium quality control sample

MeOH – methanol

ME – matrix effect

MiOH – 1'-hydroxymidazolam

MiOH-D_4 – 1'-hydroxymidazolam-D_4

MS/MS – tandem mass spectrometry

PFP – pentafluorophenyl

PPT – protein precipitation

RF - radiofrequency

QC – quality control

QqQ – triple quadrupole

s/n ratio – signal to noise ratio

SPE – solid phase extraction

UHPLC – ultra-high performance liquid chromatography

UGT – liver uridine diphosphate glucuronosyltransferase

ULOQ – upper limit of quantification

1. INTRODUCTION

In intensive care units, the precise administration of sedative drugs is crucial in order to avoid under- or over sedation. Both, can be very harmful for the patient causing numerous side-effects or pain and suffering during the surgical procedures. This is especially important in case of paediatric patients.

The purpose of this work is to develop and validate rapid ultra-high performance liquid chromatographic-tandem mass spectrometric method for analysis of three drugs – morphine, clonidine and midazolam, and their metabolites in blood plasma at very low concentrations.

The challenges for this work is small sample volume, because the patients are underage, very low drug concentrations in samples, as well as difficulties of complex and varied matrix. All analytes have basic properties (e.g. pK_a values), thus achieving chromatographic separation is challenging, especially morphine's metabolites (morphine-3-glucuronide and morphine-6-glucuronide), that have exactly the same m/z and similar fragmentation patterns for mass spectrometric detection.

In order to use the method for the real samples, it has to be fully validated and numerous validation parameters has to be evaluated. Such as selectivity, carry-over, lowest limit of quantification, calibration curve, accuracy, precision, matrix effect (including recovery and process efficiency) and stability in different conditions and time periods.

The application of the current bioanalytical method will help to understand and evaluate the pharmacokinetics of sedative drugs used in paediatric patients under the EU FP7 project CloSed – "Clonidine for Sedation of Paediatric Patients in the Intensive Care Unit". Aim of the CloSed project (phase III clinical trial) is to improve the sedation for paediatric patients and change the current dosing regimen.

2. LITERATURE OVERVIEW

2.1. Sedative drugs

Sedation is commonly used in the intensive care unit (ICU) for a number of reasons – to alleviate distress, pain and to facilitate invasive procedures. Various drugs are used for pharmacological sedation and are broadly classified into three groups [1]:

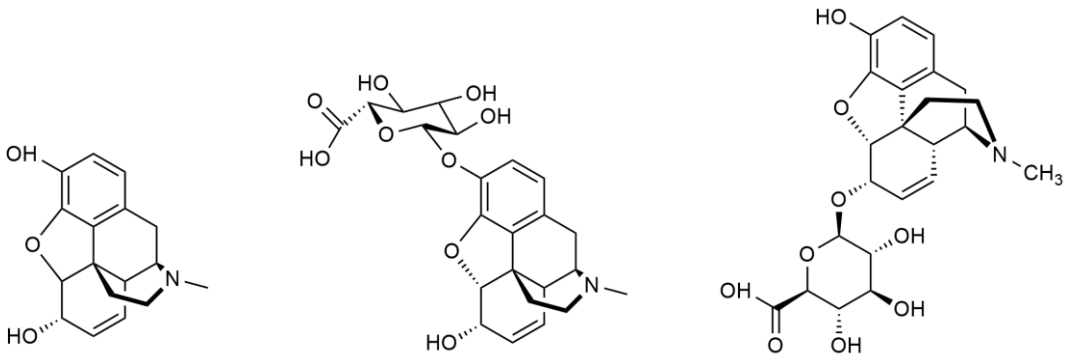
1. Analgesic drugs – primarily opioids and ketamines;
2. Hypnotic drugs – propofol and benzodiazepines;
3. Alpha-2 agonists – clonidine and dexmedetomidine.

The common practice is to use an opioid in combination with either propofol or a benzodiazepine. Sedative drug monitoring is necessary to ensure effects of the drugs, dosage regimes and, if needed, adapt the medical health care for each patient. Benzodiazepines are cheap, however there are concerns over the increased risk of delirium and lengthier stay in ICU. Furthermore, there is a potential that benzodiazepines cause renal dysfunction and accumulate in liver [1].

One of the most important issues in intensive care medicine is the determination of an individual analgosedation profile for each patient to reduce morbidity and shorten duration of therapy. The latest guidelines recommend light sedation [2]. It is a careful balancing act aimed at reducing the adverse consequences of over- and undersedation. It is recognized that overdosage could increase the incidence of pneumonia, delirium (syndrome of acute onset characterized by a fluctuation of mental state), insomnia, psychic post trauma and the expenses of hospitalization, but undersedation may include pain, anxiety and post-traumatic stress disorder [1].

2.2. Morphine

The chemical structure and monoisotopic mass of morphine ((5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol) and of its metabolic derivatives is shown in figure 1. Compound structure determines the effects observed – analgesic (pain relieving) and side effects, as well as the substance's ability to cross the blood-brain barrier [3]. Morphine is metabolized via liver uridine diphosphate glucuronosyltransferase (UGT) enzymes. Main metabolites – morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) account for 10 % of all the metabolites formed [4]. Both are very hydrophilic [3]. In negligible amounts also normorphine and hydromorphone are created [4]. Only bioactive M6G is able to pass the blood-brain barrier, and its analgesic effect is equal to or even greater than the effects of morphine [3,4].



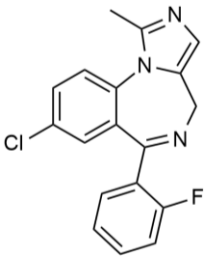
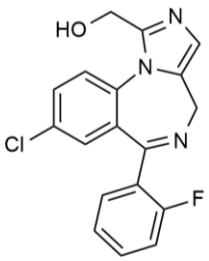
Morphine	M3G	M6G
$C_{17}H_{19}NO_3$, monoisotopic mass =285,14	$C_{23}H_{27}NO_9$, monoisotopic mass =461.17	$C_{23}H_{27}NO_9$, monoisotopic mass =461.17

Figure 1. Structures and monoisotopic masses of morphine and its metabolites.

Morphine is highly addictive sedative drug. The addiction is influenced by frequent administration of opiates and the need of the increase of the dose for the central nervous system [4]. Changes from excessive consumption occurring during development of limbic and dopaminergic system will have a lasting effect on areas in brain that regulate multiple things, including learning throughout adult life [3]

2.3. Midazolam

Midazolam (Figure 2) is a widely used short-acting benzodiazepine with hypnotic, anticonvulsant, sedative, muscle-relaxant and anxiolytic (anxiety preventing) properties, thus is used in induction of anaesthesia in ICU [5]. It has low acute toxicity and in clinical practice is administered intravenously and intramuscularly to treat generalized seizures and muscle spasms. Midazolam is currently the sedative of choice of both general anaesthesia and procedural sedation because of its rapid onset, short acting and context sensitive half-life, elimination half-life being 1.5–2.5 h [2,5]. Midazolam is hydroxylated by CYP3A4 to its primary active metabolite – 1’-hydroximidazolam (MiOH) (Fig. 2) [5].

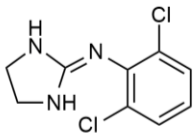



Midazolam	MiOH
$C_{18}H_{13}ClFN_3$, monoisotopic mass =325.08	$C_{18}H_{13}ClFN_3O$, monoisotopic mass =341.07

Figure 2. Structures and monoisotopic masses of midazolam and its primary metabolite.

2.4. Clonidine

Clonidine (N-(2,6-dichlorophenyl)-4,5-dihydro-1H-imidazol-2-amine) (Figure 3) stimulates alpha (2)-adrenoceptors in central nervous system which results in lowering blood pressure and decreasing of heart rate [6]. Because of that clonidine is used as an antihypertensive drug, but it has multiple other uses such as sedation and analgesia [7]. In children, clonidine is mainly used as a sedative in combination with benzodiazepines and to reduce withdrawal due to prolonged administration of sedative drugs in ICU. In adult patients clonidine may be used to provide stability during operations and to prevent symptoms of opioid and alcohol withdrawal [8]. However, there can be withdrawal reactions from clonidine itself which consist of nervousness, increased heart rate and rise in arterial pressure [6].



Clonidine

$C_9H_9Cl_2N_3$, monoisotopic mass =229.02

Figure 3. Structure and monoisotopic mass of clonidine.

2.5. Liquid chromatography-mass spectrometry (LC-MS)

High-performance liquid chromatography (HPLC) is an analytical technique widely used for the analysis of biomolecules, pharmaceuticals, and many organic compounds. HPLC is a modern form of liquid chromatography (LC) that uses small particle columns through which the mobile phase is pumped at high pressure. Reversed-phase (RP) chromatography is the most

common separation mode in HPLC, it is suitable for the analysis of water-soluble, medium-polarity, and some nonpolar analytes. The RP-HPLC separation is based on analytes' partition between a polar mobile phase and a hydrophobic (nonpolar) stationary phase. The earlier stationary phases have been replaced by more strongly bonded groups, such as C18 (octadecyl) bonded groups on silica. Polar analytes elute first while retention of less polar analytes is stronger on the C18 groups. RP-HPLC typically uses polar mobile phases such as a mixture of MeCN or MeOH with water [9].

2.5.1. HPLC and UHPLC

To further increase chromatographic resolution and throughput, ultra-high performance liquid chromatography (UHPLC) has become commercially available in 2004. It involves the use of columns packed with sub-2 μm particles at pressures up to 1300 bar (130 MPa), although differentiation from HPLC starts at separations that are achieved at pressures above 400 bar (40 MPa). In addition to increase in the chromatographic resolution, a significantly reduction in analysis time and solvent and sample consumption are possible. Because of benefits, new UHPLC methods are often developed, whereas HPLC methods can be quite easily transferred to UHPLC [10].

2.5.2. Electrospray ionization

Electrospray is a phenomenon, where liquid is "pulverized" into fine mist of highly charged droplets by application of high voltage. Since 1980'ies electrospray has been used in mass spectrometry to transfer ions from solution into gas phase. In the beginning, the electrospray ionization (ESI) was used for protein analysis and only later it was extended to smaller, polar molecules. It was also discovered that ESI was easily coupled to HPLC [11]. But conventional ESI works best if flowrates are kept between 1-20 μL/min, which is not directly compatible with flow rates used in HPLC [12].

General idea of ESI is application of a strong electric field to a liquid meniscus, which is passing through a capillary. This field induces a charge accumulation at the liquid surface. At high enough potential (2-5 kV), the liquid surface deforms into a cone (Taylor cone) and a jet of droplets evolves from the tip of the cone. The droplets then pass through heated inert gas (usually nitrogen). These highly charged, droplets will continue to lose solvent by evaporation and reduce in size. When the repulsive force between ions overcomes the surface tension of the droplet (Rayleigh limit) the Coulomb explosion occurs, i.e. subdivision of the droplet. Fully desolvated ions result from field desorption from the charged droplet or complete evaporation of the solvent. Small molecules mostly produce singly charged ions. ESI produces ions not only

by (de)protonation but also through the formation of sodium, potassium, chloride, ammonium, acetate or other adducts [11].

Pneumatically assisted ESI. In order to accommodate higher liquid flow rates nebulizing gas (usually nitrogen) is supplied coaxially with liquid capillary. As a result, liquid flow rates up to 1 ml/min can be used although highest efficiency is observed in the flow range of 10-200 µL/min. Nowadays most measurements are performed using this type of ESI [12].

On-axis and orthogonal spray. In the early models of ESI sources, the liquid capillary was aligned in-line with respect to MS entrance. However, when higher liquid flow rates are used excessive amounts of neutral solvent molecules enter the mass spectrometer and contamination is a concern. In order to avoid those issues, sprayer needle was positioned off-axes with respect to MS entrance. Configurations, where sprayer is positioned at right angle – orthogonal ESI – have appeared. In contemporary LC-MS instruments off-axis, orthogonal and double orthogonal configurations are commonly used [12].

Agilent JetStream Technology® is orthogonally aligned pneumatically assisted ESI interface with additional sheath gas flow. This super-heated (up to 400°C) sheath gas is supplied coaxially with pneumatically assisted ESI sprayer and it facilitates desolvation of ions and confines the spray. As a result 5-10 fold improvement of at generally used flow rates are observed. This ESI is recommended for analysis of pharmaceutical compounds, as well as trace-level contaminant analysis needed in environmental and food safety analyses [13].

2.5.3. Mass spectrometry

The capability of mass spectrometry (MS) are noteworthy among other analytical methods: it has excellent sensitivity (leading to low detection limits) and selectivity for a wide range of analytes [11]. When compared to the traditional LC detectors, the MS detector enables significantly more reliable identification. In analytical chemistry applications are oriented towards problems where detections of trace levels of difficult analytes in complex matrices are needed – like pharmaceutical, metabolism studies and food safety [14].

Mass spectrometers can detect only ionic species. Firstly, ions are produced in ion source and drawn into mass spectrometer. Inside the mass spectrometer the ions are moved by means of electric field. Pressure (vacuum) in mass spectrometer must be low enough to allow the ions to travel without collision for about one meter (approximately 0.001 Pa). Mass analyser uses electric and/or magnetic fields to separate ions according to their mass to charge ratio (m/z). Detectors determine the abundance of ions of particular m/z [11].

Nowadays the majority of analyses carried out with LC–MS use tandem mass spectrometric (MS/MS) detection. MS/MS is popular in routine analysis because it enables higher signal-to-

noise ratio and more reliable identification of the analytes [14]. MS/MS is a method involving two stages of mass analysis. Usually first analyser selects precursor ion, which undergoes fragmentation in collision cell [11,14]. Second mass analyser selects the product ions. Currently triple quadrupole mass spectrometers are the most common mass spectrometers for quantitative LC-MS/MS analysis. The triple quadrupole (QqQ) configuration indicates an instrument with three quadrupoles where the second one, indicated by a lower case "q", denotes the collision cell where the fragmentation of ions takes place [11].

Often MS signals and responses for the analyte are affected by the co-eluting endogenous matrix compounds. Analyte signal can be either suppressed or enhanced by the matrix compounds and this phenomenon is known as matrix effect (ME). Main source of ME in blood plasma samples are phospholipids [15].

2.6. Previously used LC-MS/MS methods for clonidine, midazolam and morphine analysis

To the best of our knowledge, there is no previous report of simultaneous analysis of clonidine, morphine and midazolam and their metabolites. However in [6], [16] and [17] clonidine is the only analyte quantified. The lowest limit of quantification (LLOQ) was different in the literature, but ranged from 0.01 – 5 ng/mL. For analysis mostly C18 columns are used [2], [5], [18], [6], [16], [17] but also C4 [7], pentafluorophenyl (PFP) [4] and biphenyl [19] is used as the stationary phase. Regular HPLC system is used besides two assays using UHPLC [2], [19]. Eluents used are common in LC – MeCN is preferred [2], [5], [6], [7], [16],[18] over MeOH [4], [17], [19]. Water phase is using either low (formic acid) or neutral (ammonium acetate) pH. The column temperature was maintained around the room temperature [2], [4], [5], [7], [17] only one assay used the column thermostat heated to 50 °C [18].

The overview of previously used methods is presented in the table 1.

Table 1. Literature review of previously used methods LC-MS conditions.

Stationary phase	Mobile phase	Eluting conditions	Detection	Limit of quantification	Reference
Hypersil Gold C18 column (1.9 µm, 50 × 2.1 mm ID)	MeCN and 0.1% formic acid	280 µL/min, isocratic elution. Column temperature 25°C	MS Midazolam m/z 326.10 → 291.20	Midazolam 5 ng/mL	[2]
Thermo Scientific AccuCore PFP column (2.6 µm 50 × 2.1 mm)	MeOH with 0.1% formic acid and H_2O with 0.1% formic acid	Column temperature 27 °C	MS Morphine m/z 286.1 → 165.1 m/z 286.1 → 185.0, M3G m/z 462.2 → 165.1 m/z 462.2 → 286.1 M6G m/z 462.2 → 165.1 m/z 462.2 → 286.1	Morphine 5,5 ng/mL, M3G 5,5 ng/mL M6G 5,5 ng/mL	[4]
Purospher® RP 18-e column, (5 µm, 150 ×4.6mm ID) LiChrospher® 100 RP 18-e pre-column, (5 µm, 4 × 4 mm ID)	MeCN and 10 mmol/L ammonium acetate aqueous solution	0.7 mL/min. Column temperature 27 °C	MS Midazolam m/z 326 → 291 MiOH m/z 342 → 203	Midazolam 0.1 ng/mL MiOH 0.1 ng/mL	[5]
X-Terra MS C18 column (5 µm, 2.1 mm × 150 mm). Phenomenex Security Guard C18 pre-column	MeCN and 0.1% formic acid	0.6 mL/min. Column temperature 50 °C	MS Morphine m/z 286.0 → 151.9 M3G m/z 462.1 → 286.1 M6G m/z 462.1 → 286.1	Morphine 3.5 ng/mL M3G 3.5 ng/mL M6G 3.5 ng/mL	[18]

			Midazolam m/z 326.0 → 291.1 MiOH m/z 342.3 → 203.0	Midazolam 0.5 ng/mL MiOH 2.5 ng/mL	
Hypersil Hypurity C18 column (5 µm, 50 × 4.6 mm ID)	MeCN and 2mM ammonium acetate	0.4 mL/min	MS Clonidine m/z 231.9→44.1	Clonidine 0.05 ng/mL	[6]
Thermo Electron BetaBasic C4 column (5 µm, 100 ×3mm) and corresponding pre-column (5 µm, 10 × 3.0 mm)	MeCN and 0.1% formic acid	350 µL/min Column temperature was maintained at room temperature	MS Clonidine m/z 230.10→213.10 m/z 232.10→215.10	Clonidine 0.1 µg/L	[7]
ZORBAX-XDB-ODS C18 column (3.5 µm, 30 x2.1 mm)	MeCN and 0.2% formic acid	0.2 mL/min	MS Clonidine m/z 230.0→213	Clonidine 0.01 ng/mL	[16]
Inertsil® ODS-3 column (3 µm, 3.0 × 50 mm)	MeOH and 5mM ammonium formate buffer (pH adjusted to 2.8 with formic acid)	0.25 mL/min Column temperature 30 °C	MS Clonidine m/z 231.9→44.1	Clonidine 0.01 ng/mL	[17]
Superficially porous Kinetex Biphenyl column (2.6 µm, 100 × 2.1 mm)	MeOH with 0.1% formic acid and H_2O with 0.1% formic acid	-	MS	Morphine 2 ng/mL Midazolam 5 ng/mL	[19]

2.7. Blood plasma

Biofluids such as blood plasma and urine represent highly complex matrices [20]. Whole blood contains red cells, white cells, and platelets suspended in plasma. Red cells (erythrocytes) carry oxygen, platelets (thrombocytes) are cell fragments in the blood which interact with clotting proteins to stop bleeding [21]. Plasma is the liquid portion of blood – a protein-salt solution in which blood cells and platelets are suspended. Plasma constitutes 55% blood volume and is composed of about 92% water, 7% proteins - albumin, gamma globulin, anti-haemophilic factor, and other clotting factors, 1% mineral salts, sugars, fats, hormones and vitamins. Plasma is obtained by separating the liquid portion of blood from the cells [22]. The blood composition can vary significantly between individuals and species, but also within an individual. A significant source of ME in plasma samples are phospholipids from cells. [20]

2.8. Blood plasma sample preparation for LC-MS analysis

2.8.1. Protein precipitation

The fastest and simplest method is the protein precipitation (PPT). To sample – either plasma or serum, organic solvent – usually MeOH or MeCN, is added to denaturate and precipitate the proteins. After this step centrifuge is used to acquire clear supernatant [3, 5]. However, it does not result in very clean extract. This sample preparation method is most likely to cause ion suppression in ESI, since it doesn't sufficiently remove endogenous compounds such as phospholipids, fatty acids, etc. [20]

2.8.2. Solid phase extraction

In this sample preparation method sample is loaded in solid phase extraction (SPE) cartridge which works similar way as LC column. There are two ways of action - either analytes are retained in SPE cartridge stationary phase and, after washing, retrieved from it with change of eluent or unwanted components are retained in stationary phase and analytes collected from SPE cartridge[7, 19]. In comparison with protein precipitation, extracts obtained from SPE are cleaner. Both reversed-phase and cation exchange SPE results in lower phospholipid levels, a significant source of ME in plasma samples, compared to PPT. However even if matrix clean-up is more extensive with SPE, the pre-concentration step increases the concentration of the target analyte together with the concentration of not removed interfering substances from the biofluid and/or the sample preparation. [20]

2.8.3. Liquid–liquid extraction

In LLE one liquid is immobilized in a tube and the other (immiscible) liquid phase is pushed through the stationary liquid in a way similar to chromatography. Extraction of analyte occurs during the contact between the two immiscible phases. The solvent moves through the packing

due to the gravity or by use of vacuum. LLE often yields clean extracts, but the procedure is quite inconvenient and time consuming. To achieve higher cleanliness and bigger analyte recovery multiple extraction steps are required [20].

2.9. Previously used sample preparation methods for clonidine, midazolam and morphine analysis

Amount of sample varied greatly between reviewed literature methods – starting from 50 µL up to 1 mL. For the sample preparation PPT was used with MeOH and MeCN [2], [4], [7], as well as combination of PPT and Dispersive Liquid Liquid Microextraction (DLLME) [19], but also acidification of MeOH using perchloric acid ($HClO_4$) [16] and alkalization with NaOH before LLE was used [5], [17]. Moreover, the on-line SPE [18] and divinylbenzene polymer in regular SPE cartridge format [6] was used for the sample preparation. More than half of articles used sample preconcentration after the sample preparation. Injection volumes varied from 10 - 20 µL and even 40 µL [7], the UHPLC methods [2] used 2 µL, but [19] used 10 µL.

The overview of previously used sample preparation methods is presented in table 2.

Table 2. Literature review of previously used sample preparation methods for LC-MS.

Analyte/matrix	Amount of the sample	Sample preparation	Concentrating	Injection volume	Reference
Midazolam/ Human blood serum	100 µL	To 50 µL of blood serum IS and 300 µL MeCN was added. Samples were vortexed for 30 s and centrifuged for 10 min at 15,800 x g.	-	2 µL	[2]
morphine M3G M6G/ Blood serum	50 µL	500 mL of the MeOH containing IS added to 50 µL blood serum. Solution was vortexed for 30 s and centrifuged for 5 min at 12,000 x g.	Supernatant was transferred to a 96-well plate, evaporated to dryness with a 60 °C air stream. Then sample was reconstituted in 500 µL 0.1% formic acid solution.	15 µL	[4]
Midazolam MiOH / Human plasma	1.0 mL	To 1.0 mL human plasma sample were added with 25 µL IS (0.1 µg/mL clobazam solution) and alkalinized with 100 µL 0.1 mol/L sodium hydroxide solution. Midazolam and MiOH were extracted from plasma samples with 4.0 mL toluene–isoamyl alcohol by shaking for 30 min. Sample was centrifuged for 5 min at 2000 x g.	Organic phase was collected and evaporated to dryness in a centrifugal evaporator vacuum system. The samples were reconstituted with 50 µL acetonitrile–10 mmol/L ammonium acetate and vortexed.	20 µL	[5]
Morphine M3G M6G Midazolam MiOH/ Human plasma	150 µL	On-line SPE was used to extract compounds and IS from plasma samples. Samples were mixed with 150 µL of IS, dissolved in ammonium acetate buffer (50 mM, pH 9.25), and shaken for 5 min.	-	10 µL	[18]
Clonidine/ Human plasma	0.5 mL	Samples were vortexed. 50 µL IS was added and vortexed again for 10 s. The samples were centrifuged for 5 min at 15,000 rpm (at 10 ºC). Samples were transferred on Orochem DVB-HL cartridges (already preconditioned	Samples was evaporated to dryness under nitrogen at 50 ºC. The samples was reconstituted with 300 µL mobile phase.	10 µL	[6]

		with 1 mL of MeOH followed by 1 mL of 2 mM ammonium acetate). Then plasma was drained out and cartridges were washed with 1 mL 10% MeOH in water. After drying, elution was carried out with 1 mL MeOH.			
Clonidine/ Blood serum	200 µL	Samples were vortexed for 15 s and centrifuged for 5 min at 15 800 × g at 4 °C. 200 µL of serum and 50 µL IS were mixed with 500 µL MeCN. Sample was vortexed for 30 s and centrifuged for 10 min at 15,800 × g (at 4 °C).	Samples was evaporated to dryness in an evaporation concentrator for 2 h at 55 °C and reconstituted with 80 µL of eluent.	40 µL	[7]
Clonidine hyrdochloride/ Blood plasma	0.1 mL	PPT with MeOH and perchloric acid ($HClO_4$) was performed. Sample was transferred into a 1 ml centrifuge tube, 0.1 mL MeOH and 0.1 mL perchloric acid were added. Sample was vortexed for 2 min and centrifuged for 20 min at 15400 rpm.	-	20 µL	[16]
Clonidine/ Human plasma	0.5 mL	To sample IS solution was added. To this 100 µL of 0.1 M sodium hydroxide was added. After vortex mixing for 10 s, a 5 mL aliquot of the extraction solvent, diethyl ether:dichloromethane (70:30, v/v), was added and the sample was vortex-mixed for 5 min.	The organic layer was transferred and evaporated to dryness using an evaporator at 40°C under a stream of nitrogen. Then the sample was reconstituted in 250 µL of eluent.	10 µL	[17]
Clonidine Midazolam/ Whole blood	0.5 mL	To sample (previously spiked with IS), was added 500 µL MeOH. After centrifugation, 500 µL of the supernatant were transferred into tube with 1 mL of water, 0.2 g of NaCl and 100 µL of saturated carbonate buffer (to reach pH=9). DLLME was performed. After that sample was shaken for 1 min (with an ultrasonic water bath) and after centrifugation the organic phase was transferred into a vial.	Sample was evaporated to dryness under a gentle nitrogen stream and reconstituted in 100 µL of eluent.	10 µL	[19]

3. EXPERIMENTAL

3.1. Chemicals and reagents

Standard substances and their respective stable isotope labelled internal standards (IS): M3G, M6G, morphine, clonidine, MiOH, midazolam, M3G-D_3, M6G-D_3, morphine-D_6 and MiOH-D_4 were obtained from Cerilllant (Texas, USA). Clonidine-D_4 and midazolam-D_6 were obtained from Toronto Research Chemicals Inc (Toronto, Canada).

Other reagents used: UHPLC-MS grade purity MeOH from Sigma Aldrich (Missouri, USA), LC-MS grade purity acetic acid from Sigma Aldrich (Missouri, USA), LC-MS grade purity ammonium hydroxide solution from Sigma Aldrich (Missouri, USA), LC-MS grade purity 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) from Sigma Aldrich (Missouri, USA), water produced with MilliQ Advantage A10 water purification system from Merck Millipore (Massachusetts, USA).

All reagent chemical and physical properties are presented in the table in Annex 1.
Human plasma was obtained from Blood Centre of Tartu University Hospital.

3.2. Preparation of standards and reagents

Eluent used for analytical method. Mobile phase A was prepared by adding 527 µL of HFIP to 999.5 mL MilliQ water. Mobile phase pH was adjusted to pH=9 with 230 µL ammonium hydroxide. Eluent was filtrated using Durapore 0.22 µm membrane filter from Merck Millipore (Massachusetts, USA).

Formic acid eluent used for cleaning the system. 0.1 % formic acid eluent was prepared by adding 1 mL of concentrated formic acid to 999 mL MilliQ water. Eluent was filtrated using Durapore 0.22 µm membrane filter from Merck Millipore (Massachusetts, USA).

Substocks and spiking the plasma. Substocks for calibration, quality control (QC) samples and IS were made from standard substances by precisely weighting the amount of standard substance and diluting them in water. Calibrators and QCs had separate substocks made from standard substances.

Calibration standards, in ten concentration levels over the range of 0.05-250 ng/mL, were prepared by spiking drug-free human blood plasma with appropriate substocks. Calibrators 50-250 ng/mL were prepared using the substock with the concentration 5 µg/mL for all analytes. Calibrators 1-25 ng/mL were prepared using the substock with the concentration 100 ng/mL for all analytes. Calibrators 0.05-0.5 ng/mL were made from the substock with concentration 10 ng/mL for all analytes.

QC samples were prepared at four concentration levels: 0.05, 0.15, 50 and 200 ng/mL by spiking drug-free human plasma with appropriate QC substock solution. Upper QC samples with 200 ng/mL concentration were made from substock, which contained all analytes in concentration 3.6 µg/mL. Middle QC samples with 50 ng/mL concentration were made from substock with concentration 0.9 µg/mL. QC samples with the concentration of 0.15 ng/mL were made from substock with concentration 2.7 ng/mL and QC samples with 0.05 ng/mL concentration were made from substock with concentration 0.9 ng/mL.

Calibrators and QC samples were aliquoted into 2 mL Eppendorf vials and stored at −80 °C until the usage. A methanol solution containing 10 ng/mL of each IS was prepared and stored at −80 °C until usage.

3.3. Sample preparation

Protein precipitation was accomplished by adding 700 µL of methanol and 50 µL methanol containing IS to 100 µL of each calibrator, QC or sample. The resulting solution was mixed for 4 min in Eppendorf MixMate mixer (Hamburg, Germany) and centrifuged at 30 000 ×g for 10 min at 4 ^{o}C Eppendorf centrifuge 5430 R (Hamburg, Germany). The supernatant (approximately 800 µL) was transferred to a 2 mL Eppendorf polypropylene vial and evaporated to dryness using Jouan RC 10-09 centrifugal evaporator (vacuum concentrator) Thermo Fisher Scientific (Massachusetts, USA) and reconstituted in 80 µL of water and methanol mixture (8:2, v/v). An aliquot of 6 µL was injected to the UHPLC-MS/MS system.

3.4. HPLC conditions

Agilent 1290 Infinity (Santa Clara, USA) UHPLC system consisted of binary pump, heated column compartment and autosampler. Autosampler temperature was set at 4 °C. Analytes were separated using Waters Acquity UPLC BEH C18 (1.7 µm particle size, 2.1 × 100 mm) analytical column (Milford, USA) with Waters VanGuard BEH C18 (1.7 µm particle size, 2.1 × 5 mm) pre-column (Milford, USA), which were maintained at 30 °C. Agilent in-line filter with 0.3 µm frit (Santa Clara, USA) was also used. Mobile phase consisted of water containing 5 mM HFIP (v/v) (at pH 9) (mobile phase A) and methanol (mobile phase B).

The analytes and their respective internal standards were eluted using a gradient elution (Table 3) with the duration of 7 minutes. An additional 3 min post run with isocratic elution of the mobile phase B at 5 % was used in order to condition the column after the analytical run. The retention times for the analytes ranged from 3.5 min to 6.06 min (Table 4).

Table 3. Eluent gradient to obtain the chromatographic separation.

Time, min	Eluent composition 5 mM HFIP (pH 9)/ MeOH, %
0	95/5
1.0	95/5
3.7	25/75
5.8	25/75
5.9	0/100
6.9	0/100
7.0	95/5

3.5. Mass spectrometric parameters

Quantification of the analytes and internal standards was achieved with Agilent 6495 Triple Quad mass spectrometer (Santa Clara, USA), equipped with an Agilent JetStream electrospray ionization source. The instrument was operated in positive ionization multiple reaction monitoring (MRM) mode. Agilent MassHunter Quantitative Analysis software version B.07.00 was used to quantify the analytes.

The following mass analyser settings were used: gas temperature (135 °C), gas flow (13 L/min), nebulizer (25 psi), sheath gas temperature (400 °C) and sheath gas flow (11 L/min), capillary voltage (2500 V) and nozzle voltage (500 V). iFunnel parameters at high pressure were 210 V and at low pressure 220 V

Optimized collision energies for each analyte and internal standard are listed in table 4. Product ions were selected based on both ion abundance and consistency in fragment ion formation over multiple infusions. The most abundant and consistent fragment ion was used for analyte detection. Measurement of the analyte concentrations was based on the specific analyte/IS peak area ratio.

Table 4. Retention times, recorded transitions and respective collision energies (CE) for all the analytes and IS-s.

Analyte	Retention time, min	Precursor ion, *m/z*	Quantifier, m/z	Quantifier CE, V	Qualifier, m/z	Qualifier CE, V
M3G	3.50	462.2	286.0	26	200.8	46
M3G-D_3	3.50	465.2	289.0	32	-	
M6G	3.95	462.2	286.0	32	200.8	52
M6G-D_3	3.95	465.2	289.1	36	-	
morphine	4.80	286.2	152.0	64	165.1	49
morphine-D_6	4.80	292.2	152.0	64	-	
clonidine	4.91	230.0	44.0	29	212.9	28
clonidine-D_4	4.91	234.1	48.2	32	-	
MiOH	5.70	342.1	203.0	29	324.0	21
MiOH-D_4	5.70	346.1	202.9	29	-	
midazolam	6.06	326.1	291.1	29	222.0	57
midazolam-D_6	6.06	332.1	297.0	32	-	

4. RESULTS AND DISCUSSION

4.1. Assay development for the simultaneous determination of clonidine, midazolam, morphine and their metabolites from the blood plasma

Assay development contained two major parts:

I **LC-MS method development –** including suitable conditions for the sample preparation, mobile phase composition to obtain the separation between the analytes and suitable parameters for the MS detection.

II **Method validation –** including the estimation of the linear range, limit of quantification, method within-day and between-day accuracy and precision evaluation. Evaluation of the ME, process efficiency, recovery, selectivity and analyte stability under different storage conditions and temperatures.

4.2. LC-MS method development

4.2.1. Sample preparation for blood plasma

4.2.1.1. Solid phase extraction

Solid-phase extraction with Phenomenex Phree phospholipid removal plates (Torrence, CA, USA) were tested and difference between the Phree and PPT sample preparation techniques was evaluated by comparing compounds peak areas.

Phree stationary phase contains zirconium oxide that helps to selectively bind phospholipids [23]. Phree yielded lower recoveries (by comparing peak areas) for all analytes compared to protein precipitation for all compounds – especially for M6G when using Phree resulted in 95 % decrease in peak areas. For the rest of the compounds and internal standards decrease was observed as well – for M3G by 79 %, for morphine 65 %, for clonidine 59 %, MiOH 28 % and for midazolam by 72 %. Extended table can be found in Annex 2.

Even if Phree SPE removes phospholipids, it also removed polar glucuronides, resulting in poor recoveries for the analytes. Therefore, even if SPE with Phree phospholipid removal cartridges results in cleaner extract, the low LLOQ levels required for the clinical trial were not achieved.

4.2.1.2. Protein precipitation

Three eluent mixtures were tested – pure MeOH, MeOH and MeCN in ratio 1:1 and MeCN. MeOH and MeCN mixture gave the worst results. Results obtained from MeCN varied and did not result in consistent peak areas. PPT using MeOH resulted in satisfactory recoveries and thus decided to take it in use.

In the beginning PPT was done with plasma:MeOH in ratio 1:6 and after precipitation supernatant was diluted 1:1 with H_2O. To increase the sensitivity of the method, amount of organic solvent was decreased to final ratio 1:2, which lead to disappearance of morphine peak. Morphine most likely co-precipitated with proteins during sample preparation.

Required LLOQ was achieved using sample concentration after the PPT. Evaporation under nitrogen stream was tested, but resulted in inconsistent results, thus vacuum concentrator centrifugal evaporator was chosen. To avoid contaminating the chromatographic column with matrix both in-line filter and pre-column are used.

4.2.2. Selection of the suitable eluent and achieving the chromatographic separation

Suitable eluent. All compounds analysed are basic (M3G pK_a = 9.17 [24], M6G pK_a = 9.12 [25], morphine's pK_a = 8.21 [26], clonidine's pK_a = 8.16 [27], MiOH pK_a = 4.99 [28] and midazolam pK_a = 6.57 [29]), as well as the eluent water phase additive HFIP (pK_a = 9.3 [30]), thus the eluent was adjusted to pH = 9. Note that M3G and M6G have carboxylic acid functional group in their structure, which is deprotonated (ionized) at the used pH. If pH of the medium is higher than pK_a of the basic analyte then the analyte is predominantly in its deprotonated form; if pH < pK_a then basic analyte is in protonated form; if pH = pK_a then half of analyte molecules are protonated. If molecule isn't protonated (charged), it's less polar and thus retains more strongly on stationary (non-polar) C18 phase, increasing the compound's retention time. If molecule is charged, it is polar and it elutes faster under reversed phase chromatographic conditions.

HFIP was added to enhance compounds signal in the mass spectrometric detection [31] and to separate chromatographically M3G and M6G, which is essential since M3G and M6G have equal m/z and similar fragmentation pattern. HFIP acts as a weak ion-pairing additive in the basic mobile phase and therefore provides alternative selectivity in C18 stationary phase [32]. MeOH was chosen as organic phase, because eluent additive HFIP is immiscible with MeCN.

Chromatographic separation.

Several gradient programs were tested with the same eluent composition to obtain the separation between the compounds.

Various gradient programs were tested and it was observed, that:

- to prevent glucuronide elution at the very beginning of chromatogram and to ensure peak separation, MeOH content has to be slowly raised from 5 % to 75 %;
- best separation for MiOH, midazolam and clonidine peaks is achieved with isocratic elution with 75% of MeOH;

- if 100% MeOH content is reached in 3.6-5.4 minutes, clonidine, MiOH and midazolam elute together;
- if 100% MeOH content is reached in 5.5 minutes, MiOH and midazolam peaks overlap.

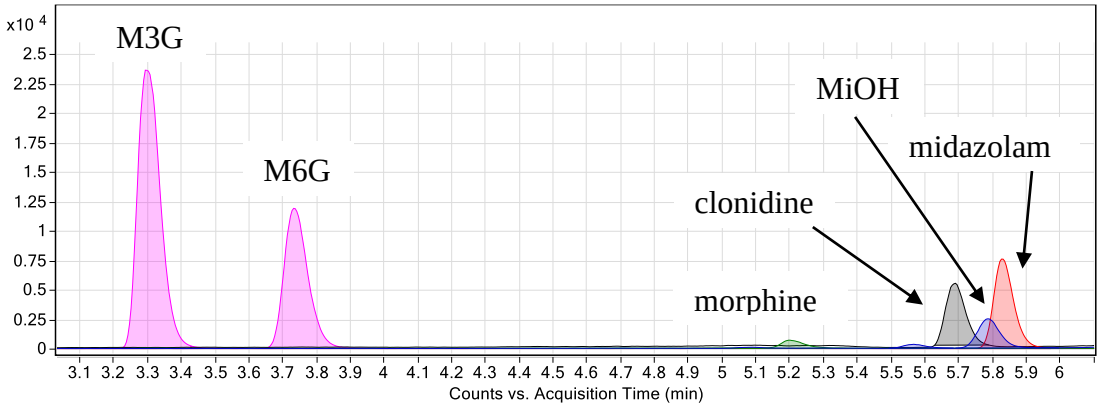


Figure 4. One of chromatograms obtained during gradient elution optimization.

One example from gradient optimization process (Figure 4) was obtained using gradient elution (Table 5), which had too steep increase of MeOH and reached 100% of MeOH content too fast, thus last 3 peaks have eluted together.

Table 5. Gradient elution optimization.

Time, min	**Eluent composition 5 mM HFIP (pH 9)/ MeOH, %**
0	98/2
1.0	98/2
3.5	49/51
4.0	20/80
5.5	0/100
6.5	98/2

Final mobile phase composition for compounds' elution was – 71% of MeOH in mobile phase for M3G, M6G elutes after 0.2 minutes with 75% MeOH content, morphine elutes after 1.1 minute with 75% MeOH content, clonidine elutes after 1.2 minutes with 75% MeOH content, MiOH elutes after 2 minutes with 75% MeOH content and midazolam elutes after 0.2 minutes with 100 % MeOH content in mobile phase.

Analytes' pK_a values are presented in the beginning of the section 4.2.2. Basic pK_a values of glucuronides are very close to the pH of eluent, therefore, half of the molecules in the mobile phase have become charged (protonated) and thus polar. As glucuronides also have carboxylic acid group, which is completely deprotonated at used pH, then the M3G and M6G molecules

are very polar and therefore elute early from the chromatographic system. Morphine and clonidine have similar pK_a values, thus their complete chromatographic separation was difficult. Since their pK_a values are lower (than those of glucuronides) and lower than pH of the eluent, less molecules have bound protons and are less polar explaining their longer retention time and stronger interaction with C18 stationary phase. MiOH is more polar than midazolam, because its pK_a comes from –OH group and, above pH = 4.99, the –OH group is deprotonated and more polar than midazolam, which binds protons only at nitrogen, and thus MiOH elutes before midazolam.

Obtained chromatogram is presented in figure 5 and compound retention times in table 4.

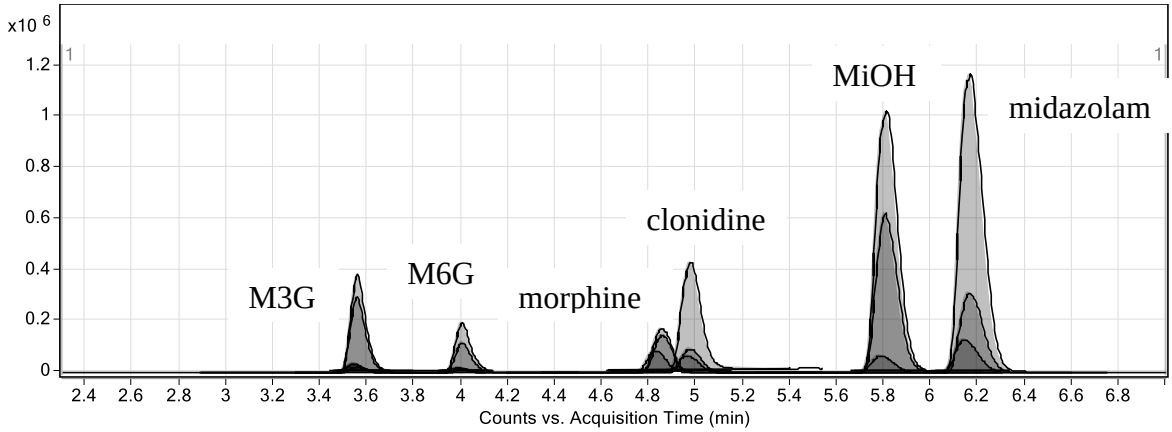


Figure 5. Achieved chromatographic separation of analytes. Different shades denote signals of quantitative and qualitative transitions and IS transitions. Concentration of all analytes in analysed sample is 200 ng/mL.

4.2.3. Additional parameters

Column temperature was maintained at 30 ºC. Different injection volumes – starting from 3 µL, until 10 µL (with step of 1 µL) were tested. From the injection volume of 7 µL, the s/n ratios for all of the compounds decreased, but after injecting 8 µL of sample, the carry-over for clonidine, MiOH and midazolam appeared. Therefore, 6 µL was chosen as injection volume for the method, which provided required LLOQ.

4.2.4. Optimization of the MS parameters

MassHunter Workstation Software Optimizer for 6400 Series Triple Quadrupole Version B.07.00 was used to find MRM transitions and collision energies (CE) for each compound. MassHunter Workstation Software Source and iFunnel Optimizer for 6400 Series Triple Quadrupole Version B.07.00 was used to optimize High Pressure RF (optimization range: 70-210 V), Low Pressure RF (40-160 V), Gas temperature (120-230°C), Gas Flow rate (11-20 l/min), Nebulizer gas pressure (20-40 psi), Capillary voltage (1500-4500 V).

4.3. Method validation

The method validation was performed according European Medicines Agency guideline [33].

4.3.1. Selectivity

During validation 6 independent blank plasma samples were analysed. To re-assure the selectivity, analysis of the double blank plasma sample (blank plasma without the addition of internal standard during the sample preparation) is conducted every time when calibration samples are analysed. Representative chromatogram can be seen in figure 6. Peak seen at 4.4 min in the transition of M6G-D3 does not interfere the analysis since the retention time for M6G-D3 is different (3.95 min). Contamination peak retention times differ greatly and it cannot influence other analytes, because analysis is done in MRM and analyte m/z are different.

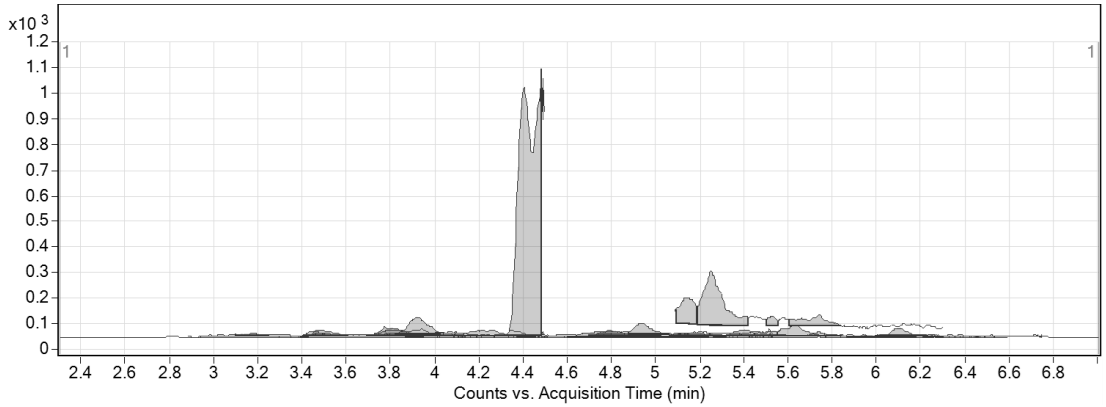


Figure 6. Representative chromatogram of the double blank plasma injection.

4.3.2. Carry-over

Carry-over was evaluated by comparing 50 pg/mL and ULOQ sample peak areas to blank injection peak areas. After the injection of higher concentration samples (e.g. 50 ng/mL) carry-over was observed.

Methods tested for carry-over reduction are described in Table 6 and expanded results for the improvement of the carry-over are presented in Annex 3. Multiple blank injections and pro-longed washing (methods A and B) with 100% MeOH did not decrease the carry-over sufficiently. Injection of 0.1 % formic acid solution decreased carry-over significantly (method C), but not sufficiently enough, thus it was decided to completely change eluent A to 0.1 % formic acid solution for washing program. Acidic eluent was chosen because analytes are basic (for pK_a's see section 4.2.2.). In acidic conditions basic analytes become protonated, thus become polar and less retained to the non-polar C18 stationary phase. Further various modifications of washing programs were tested (methods D-H in table 6.).

Method D proved that washing with 0.1 % formic acid as eluent A compared to the basic eluent (methods A and B) is more efficient. Sample (with concentration 50 ng/mL) was injected and run with method D to detect the eluent composition in acidic conditions for analytes' elution. Obtained knowledge was used to improve the washing and methods E to H were created and tested.

Methods F and G were tested with the hope that rapid increase of MeOH % in mobile phase will wash the analytes out. No improvement was obtained using these methods and carry-over in the second blank injection remained high (34 – 136 % for different analytes using method G). The increase of the carry-over in the second blank injection can be explained with the insufficient column conditioning and analyte contamination also in the needle. In order to further clean the system, the needle wash with MeOH and 0.1 % formic acid 1:1 (v/v) was conducted.

Overall best results were obtained with the methods E and H, even if carry over was slightly larger for MiOH and midazolam compared to methods F and G. After two consecutive injections with the method H, the carry-over was sufficiently low for all analytes (0 %).

Due to low concentration of IS, no carry-over was observed for them.

Table 6. List of methods used to reduce the carry-over.

Method	Description
Method A	General analytical method using HFIP and gradient elution, as described in table 3
Method B	Gradient elution using HFIP (as described in Table 3) with prolonged washing with 100% of MeOH. Total runtime 28 min.
Method C	Method B with 20 µL 0.1 % formic acid injection.
Method D	Gradient elution using 0.1% formic acid and increasing slowly MeOH content to 100 % over 20 minutes.
Method E	Gradient elution using 0.1% formic acid and MeOH. MeOH content was raised in increments of 5, 15, 30, 60, 100 % and back to 5 % over 26 minutes.
Method F	Gradient elution using 0.1% formic acid and MeOH. MeOH content was raised to 100% twice during 10 min.
Method G	Gradient elution using 0.1% formic acid and MeOH and increasing MeOH content to 100% twice during 20 min.
Method H	Gradient elution using 0.1% formic acid and MeOH. MeOH content was raised from 5 % increments of 15, 80 and 100 % over 28 min. 10 µL 0.1 % formic acid was injected.

Precise eluent gradient for method H can be seen in table 7. After each run column is conditioned with 5 min post run using 95 % of 5 mM HFIP as an eluent A.

The washing step (if needed, then multiplied) ensures the carry-over rate of less than 20 % of signal in LLOQ sample for the next injection for all analytes [33] and less than 5 % for internal standard signal.

Table 7. Eluent gradient for 0.1 % formic acid wash to reduce carry-over.

Time, min	0.1% formic acid / MeOH, %
0	95/5
5.0	95/5
7.0	85/15
10.0	85/15
15.0	20/80
20.0	20/80
22.0	0/100
27.0	0/100
28.0	95/5

4.3.3. Lower limit of quantification.

The target LLOQ levels were achieved with s/n 5 or higher and LLOQ level accuracy and precision were within 20% – as required by EMEA guideline [33]. As well there is danger of carry-over with lower levels of analyte. LLOQ was additionally assessed by using accuracy and precision. LLOQ's achieved with their respective s/n ratios are listed in table 8.

Table 8. The lower limit of quantification and signal to noise ratio for all analytes.

Analyte	Achieved LLOQ, pg/mL	S/n ratio
M3G	55	5.4
M6G	53	6
morphine	55	5.5
clonidine	55	28.7
MiOH	55	96.5
midazolam	49	94

4.3.4. Calibration curve

Matrix matched calibration consisted of 10 concentration levels in addition to double blank (sample without analytes and internal standard) and blank (sample containing only internal standards) samples and was analysed in duplicates. Range 0.05–250 ng/mL was chosen to fit the expected concentrations in the clinical trial samples. Curve was constructed using weighted least squares-fitted linear regression and squared regression coefficient for all analytes was bigger than 0.9930 (Table 9). Weighing of each analyte calibration curve was applied, based on better fit and due to the fact that, if applicable weighting factor $1/x^2$ should be used for all

bioanalytical LC-MS/MS assays [34]. All calibration curve points were within set accuracy limits of 85-115% [33] of their back calculated values.

Table 9. Calibration curve parameters for all compounds.

Analyte	Slope	Intercept	Weighting	R^2
M3G	0.3952	0.0023	1/x	0.9992
M6G	0.3636	0.0070	$1/x^2$	0.9935
morphine	0.0383	0.0008	1/x	0.9989
clonidine	0.1575	0.0001	$1/x^2$	0.9969
MiOH	0.1948	0.0006	$1/x^2$	0.9939
midazolam	0.19995	0.0006	$1/x^2$	0.9930

4.3.5. Accuracy

Accuracy describes the closeness of the analytical result to the nominal concentration of the analyte [33]. Accuracy was evaluated with QC samples at four levels with 5 samples at each level in every run. QC samples were spiked using separate stock solutions with appropriate dilutions and quantified using the calibration curve.

Accuracy is influenced both by analyte and concentration level – at low concentrations, when noise level is more influential and peak shapes is often not ideal, the range for the accuracy is wider. This is especially true at LLOQ level for M3G (97-111 %), clonidine (100-113 %) and midazolam (87-101 %). Morphine's between day accuracies vary in the wide range (the poorest being at medium QC (MED) level 95-107 %) for all concentration levels due to tailing peak shape, however in general highest accuracies for analytes were obtained in MED concentration level (50 ng/mL). At high concentrations the results are generally overestimated 101 – 110 %, except for M6G which has 87 % accuracy. This is result of weighing the calibration curve and thus giving more weight to low concentration samples, but also can indicate the mismatch between calibrators and QC samples due to the spiking error.

There is no analyte which concentration is systematically underestimated. MiOH is the only analyte with overestimated concentrations (101-110 %) at all concentration levels. This can be explained again with discrepancies of QC samples and calibration curve caused by the inaccurate spiking or by possible degradation of the analyte. In general, accuracies remained within allowed range, according to guideline [33] for all of the compounds – within 15% for 3xLLOQ, MED and ULOQ level and within 20% for LLOQ level.

Between run accuracy and precision are presented in table 10 and annex 4.

Table 10. Between run accuracy and precision for all analytes.

Analyte	LLOQ, 50 pg/mL		3xLLOQ, 150 pg/mL		MED, 50 ng/mL		ULOQ, 200 pg/mL	
	Accuracy, %	CV, %	Accuracy, %	CV, %	Accuracy, %	CV, %	Accuracy, %	CV, %
M3G	102	3.9	95	4.3	96	2.4	101	2.8
M6G	99	9.6	101	5.2	91	5.5	87	2.5
morphine	102	4.3	101	3.6	99	2.7	102	3.2
clonidine	108	3.7	95	2.1	95	1.8	108	2.8
MiOH	104	3.4	102	2.1	106	2.2	110	2.7
midazolam	93	2.6	89	1.5	89	1.6	110	2.8

4.3.6. Precision

Precision describes the closeness repeated individual measurements of analyte and is expressed as the coefficient of variation (CV) [33], which was calculated using formula (1).

$$CV = \frac{\sigma}{\mu} \times 100\%, \quad (1)$$

where σ is the standard deviation and μ the mean value of the measured concentration.

Similarly to accuracy, also precision depends on analyte and concentration level and low concentrations are influenced and vary more, especially M6G with CV being 9.6 % at LLOQ level, which is also the largest among all the analytes at all concentration levels. This is due to high noise level at low concentrations of glucuronides. In general precision remained within allowed range, according to guideline [33] for all of the compounds – below 15% for 3xLLOQ, MED and ULOQ level and below 20% for LLOQ level. Extended table can be seen in table 10 and in annex 4.

4.3.7. Matrix effect, recovery and process efficiency

Matrix effects were estimated according to the validation guideline [33] and Matuszewski et al [15]. Three sample sets were analysed: (A) analytes in neat solvent (standard solution), (B) samples where analytes have been added after PPT (post extraction spike) and (C) which were regular QC samples (pre extraction spike).

Formulas used in this section for calculating are as following. For matrix effects formula (2) was used:

$$ME = \frac{B}{\mathrm{A}} \times 100\%, \quad (2)$$

For the estimation of recovery formula (3) was used:

$$Recovery = \frac{C}{\mathrm{B}} \times 100\%, \quad (3)$$

For process efficiency formula (4) was used:

$$PE = \frac{C}{\mathrm{A}} \times 100\%\,, \qquad (4)$$

Matrix effects. ME in MS detection occur due to the components of sample matrix which were not removed during sample preparation. In case of blood plasma samples – mostly phospholipids. Phospholipids have long hydrocarbon chains and get strongly embedded into the hydrophobic C18 stationary phase – thus it is impossible to predict their retention time and whether they enhance (ME over 100%) or suppress (ME under 100 %) the analytical signal. Matrix effects aren't observed if the ME value is 100% since signal response from analytes in the standard solution is the same as in plasma sample. ME were evaluated comparing analyte concentrations at three QC levels composed from post-extraction spike and standard solution, for both the IS was added.

Bigger matrix effect is observed at low concentration level, because the competition over the ionization in the ESI source is greater when analyte concentration is lower. Especially influenced are M6G and morphine at LLOQ and 3xLLOQ levels where signal enhancement is observed (121 - 153 %), but also standard deviations of results are high. It's also apparent that there is constant signal enhancement (105 – 131 %) for morphine over all concentration levels. The ME for clonidine, MiOH and midazolam at LLOQ concentration level is low (ranging from 95 to 98%), but as concentration increases, the signal enhancement with ME of 106 – 110 % is observed.

Use of IS is helping to take into account the matrix interference, since IS should mimic the ionization of the analyte. However, if any differences in retention time is observed, the ME for analyte and IS can be different.

Determined ME values and their standard deviations are shown in table 11.

Table 11. Matrix effect data for compounds in all levels.

Analyte	LLOQ, 50 pg/mL		3xLLOQ, 150 pg/mL		MED, 50 ng/mL	
	ME, %	Std, %	ME, %	Std, %	ME, %	Std, %
M3G	84	4	98	3	95	3
M6G	153	16	121	11	99	3
morphine	131	16	115	5	105	3
clonidine	95	13	110	4	109	2
MiOH	97	5	110	3	107	3
midazolam	98	3	106	2	104	1

Recovery and process efficiency. Recovery, if calculated using formula (3) is influenced already by the matrix effects, whereas process efficiency obtained with formula (4) describes combined effect of recovery and matrix effect [15]. Overall recovery for all analytes at all concentration levels range from 62 % to 93 %. There is a clear trend in recovery decrease with the increase of the analyte concentration. Usually, this could be explained with the overabundance and co-precipitation of analyte in higher concentrations (the recovery for MED is 62 – 70%). However, this hypothesis should be tested separately for the confirmation, since the MED concentration is still quite low (~50 ng/mL) and sample pre-treatment is using 7 times more MeOH than the volume of blood plasma, therefore the precipitation should not suffer with the co-precipitation effect.

Process efficiency has two very high values at LLOQ 143 % for M6G and 110% for morphine and 96% for M6G in 3xLLOQ, which can be explained with ME (as seen in Table 11). Overall process efficiency for LLOQ levels (excluding M6G and morphine) are 68 – 82%, for 3xLLOQ (excluding M6G) 68 - 80% and for MED level 64- 75 %. Extended tables for recovery and process efficiency can be found in annex 5.

4.3.8. Stability

Freeze and thaw stability. Freeze and thaw stability is evaluated in order to take into account the possible interferences and accidental thawing during the sample transportation. For the evaluation of the freeze and thaw stability, spiked plasma samples at three concentrations (LLOQ, 3xLLOQ and MED) were frozen at -80 ºC freezer and thawed at room temperature, in three cycles. At each cycle, the samples were kept at -80 ºC for at least 24 h. The biggest influence from repeated freezing and thawing are on analytes at low concentrations, but there is almost no influence if analytes' concentration is higher than 50 ng/mL. The biggest degradation occurs at LLOQ level for morphine (only 76 % of initial concentration) and M6G – 88 % of initial concentration, however morphine is the only analyte which exceeds 80-120 % limits set by guideline [33] of change in concentration, as can be seen in figure **7** and extended table in annex 6.

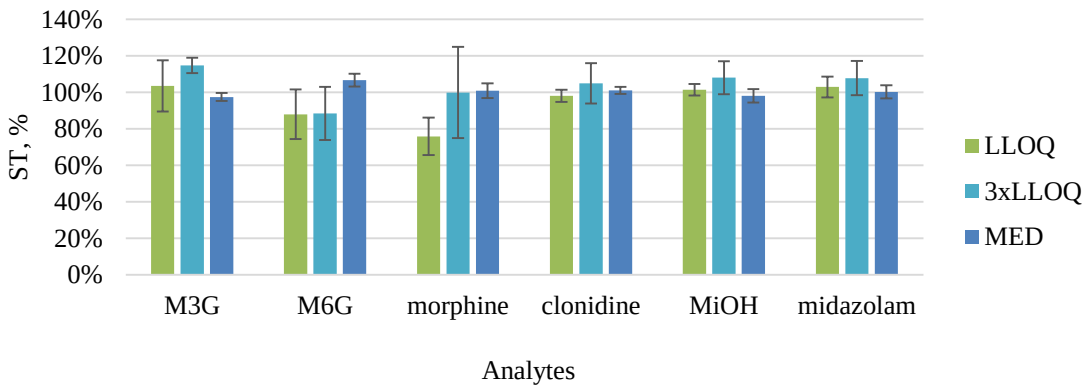


Figure 7. Freeze and thaw stability for all analytes over three concentration levels (50 pg/mL, 150 pg/mL and 50 ng/mL).

Short term stability or bench-top stability. For the evaluation of the bench-top stability spiked plasma samples at four concentrations (LLOQ, 3xLLOQ, MED and ULOQ) were kept at the monitored room temperature (22 ºC±1 ºC) for 24 hours. Variations for all analytes at all four concentration levels were within 85-105 % except for morphine at LLOQ and 3xLLOQ concentration levels. Morphine's concentration after 24 hours was 85-86 % for low concentrations levels, but the standard deviation for these results was high. Nonetheless, it is safer to decrease the required time at the room temperature before the sample preparation to minimum. Extended table with short term stability results can be seen in annex 6.

Stability in autosampler after 24 hours. For the evaluation 24 hour stability of spiked plasma samples at four concentrations (LLOQ, 3xLLOQ, MED and ULOQ). Samples were kept in autosampler with average temperature 4 ºC. Concentration variations were within 85-105 % for all compounds at all four concentration levels, however [18] suggests that morphine and its metabolites can be absorbed on glassware, thus it might not be advisable to keep it in glass vials in autosampler for longer periods of time and using polypropylene vials can improve the stability. Extended table with stability results in autosampler can be seen in annex 6.

Long term stability at -20 ºC and -80 ºC. Long term stability tests were conducted, keeping low (LLOQ 50 pg/mL) and high (ULOQ 200 ng/mL) concentration samples in -20 ºC and -80 ºC freezer in order to evaluate the most suitable storage conditions.

At LLOQ concentration level most influenced by storage was morphine - over 4 month time and storage in both at -20 ºC and at -80 ºC freezer only 70 % of analyte remained from the original concertation. For M6G the decrease in concentration was observed (larger if sample was kept at -20 ºC) and after 4 months only 77 % of M6G (at -80 ºC) remained. Change in concentrations at -80 ºC was insignificant for M3G and MiOH. For clonidine and midazolam

no clear trends were observed, but degradation still occurred already after 1 month, even if samples were kept at -80 ºC.

At ULOQ concentration level all analytes maintained 85-115 % of original concentration in both -20 ºC and -80 ºC storage conditions, however standard deviations for morphine and its metabolites were significantly higher than those for clonidine, MiOH and midazolam. No clear trends were observed, however for all analytes 3rd month's -20 ºC and 4th month's -80 ºC results show the increase for all compounds, however the results still remain within the allowed accuracy and precision for the bioanalytical method. Extended table with long term stability results can be found in annex 7.

SUMMARY

A highly sensitive simultaneous UHPLC-MS/MS method was developed for the quantification of morphine, morphine-3-β-glucuronide, morphine-6-β-glucuronide, clonidine, midazolam and 1'-hydroximidazolam in human plasma samples. The lowest limit of quantification (LLOQ) for all analytes was 50 pg/mL using only 100 µL of blood plasma. For the sample preparation, protein precipitation was used. Analytes were separated chromatographically using C18 column with weak ion-pairing additive 1,1,1,3,3,3-hexafluoro-2-propanol with pH = 9 (adjusted using ammonium hydroxide) and methanol. Samples were analysed with triple quadrupole mass spectrometer in multiple reaction monitoring mode.

Method was fully validated and method was using matrix matched calibration in the range of 0.05-250 ng/mL for all analytes. Weighted least squares-fitted linear regression with R^2 bigger than 0.993 for all analytes was obtained. The carry-over was observed, but managed with special washing program using 0.1 % formic acid in the eluent with methanol. Matrix effects were evaluated for all compounds and are reckon with the usage of stable isotope labelled internal standards for every analyte. Within-day accuracy for all analytes remained 87-113 %, but within-day precision remained within 3-11 % for all analytes at all concentration levels over the calibration range. The freeze and thaw stability for all compounds remained within 88-115%, except for morphine – only 76 % left of initial concentration at LLOQ level. 24 hour stability in the autosampler at 4 ^{o}C for all compounds remained within 85-105% and bench top stability was within 88-109 % for all analytes, except for morphine which had 69 % left of initial concentration at LLOQ. Overall, the long term stability for all analytes was better at -80 ^{o}C compared to the -20 ^{o}C, therefore the samples for the clinical trial should be stored at -80 ^{o}C.

The method will be applied for real patients' samples in the EU FP7 project CloSed – "Clonidine for Sedation of Paediatric Patients in the Intensive Care Unit".

UHPLC-MS/MS metoodika arendamine ja valideerimine uinutite ning nende metaboliitide määramiseks inimese vereplasmast.

Rūta Veigure

KOKKUVÕTE

Käesoleva töö käigus töötati välja metoodika mõnede uinutite – klonidiin, morfiin ja midasolaam ning nende metaboliitide (morfiini-3-glükoroniid, morfiini-6-glükoroniid 1'-hüdroksümidasolaam) samaaegseks määramiseks inimese vereplasmast, kasutades ülikõrgefektiivset vedelikkromatograafiat koos elektropihustusionisatsioon massispektromeetriga. Sobivaimaks proovi ettevalmistuse metoodikaks osutus valkude sadestamine metanooliga ning proovi kontsentreerimise seejärel vaakumtsentrifuugi abil. Kõigi analüütide määramispiiriks sealjuures oli 50 pg/mL ning kasutatud proovi kogus kõigest 100 µL, Kromatograafiliste parameetrite optimeerimisel saavutati ainete lahutus gradientelueerimisel mobiilfaasiga, mis koosnes nõrgast ioon-paar reagendist 1,1,1,3,3,3-heksafluoro-iso-propanool (pH 9) ja metanoolist. Uinutite ja nende metaboliitide kvantitatiivseks määramiseks kasutati tandem-massispektromeetrit detekteerides iga ühendi jaoks optimeeritud molekulaariooni [M+H]+ ja nende fragmente. Metoodika kasutab iga aine jaoks isotoopmärgistatud sisestandardeid.

Metoodika täielikul valideerimisel hinnati saagist, maatriksiefekte, protsessi efektiivsust, metoodika selektiivsust, määramispiiri, lineaarsust, analüütide stabiilsust vereplasmas erinevatel tingimustel ning määramise mõõte- ja kordustäpsust.

Metoodika oli lineaarne kontsentratsioonide vahemikus 0.05-250 ng/mL kõikide analüütide jaoks (R^2 > 0.993) kasutades maatriksvastavat kalibreerimist. Metoodika valideerimise suurimaks probleemiks osutus proovide ülekandumine pärast kontsentreeritud lahuste analüüsi. Viimane lahendati edukalt kasutades efektiivset pesuprogrammi ja happelist mobiilfaasi (0.1 sipelghappe lahus). Maatriksiefekte aitas arvesse võtta isotoopmärgistatud sisestandardite kasutamine. Metoodika päevasisene mõõte- ja kordustäpsus olid vastavalt 87-113% ja 3-11% kõikide analüütide jaoks üle lineaarse ala kontsentratsioonide vahemiku. Proovide stabiilsuskatsed näitasid mõningast anaüütide langunemist erinevatel tingimustel, millega tuleb proovide säilitamisel arvestada (näiteks pikaajaline säilitamine -80 ^{o}C juures.

Metoodikat rakendatakse EU FP7 projekti CloSed – „Klonidiin sedatsiooniks lasteintensiivravi osakonnas" raames uinutite kontsentratsiooni määramiseks patsientide proovidest.

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ANNEXES

Annex 1. Chemical reagent information.

Chemical	Manufacturer	Purity, %	CAS No	Melting point [°C]	Boiling point [°C]	d [g/cm3]	Mw [g/mol]
M3G	Cerilliant	99.6	20290-09-9	-	-	-	461.46
M6G	Cerilliant	98.2	20290-10-2	-	-	-	461.47
morphine	Cerilliant	99.7	57-27-2	255	190	1.32	285.34
clonidine	Cerilliant	100.0	4205-90-7	130	319	-	230.10
MiOH	Cerilliant	99.9	59468-90-5	265	-	-	341.77
midazolam	Cerilliant	99.6	59467-70-8	158-160	-	1.36	325.77
M3G-D_3	Cerilliant	99.7	136765-44-1	-	-	-	464.48
M6G-D_3	Cerilliant	99.5	219533-69-4	-	-	-	464.48
morphine-D_6	Cerilliant	98.5	1334606-17-5	-	-	-	291.30
clonidine-D_4	Toronto research chemicals INC, Canada	98	67151-02-4	-	-	-	234
MiOH-D_4	Cerilliant	99.6	NA	-	-	-	345.79
midazolam-D_6	Toronto Research Chemicals INC, Canada	98	1246819-79-3	-	-	-	331.80
Methanol	Sigma Aldrich USA	UHPLC-MS grade, ≥99.9%	67-56-1	-98	64.7	0.791	32.04
Ammonium hydroxide solution ≥25% in H_2O	Sigma Aldrich USA	LC-MS grade	1336-21-6	-58	38	0.9	35.05
Formic acid	Sigma Aldrich USA	LC-MS grade, ~98%	64-18-6	8.2-8.4	100-101	1.22	46.03
HFIP	Sigma Aldrich USA	LC-MS grade, ≥99.8%	920-66-1	-4	59	1.596	168.04

Annex 2. SPE Phree peak area comparison.

Calibrator concentration, ng/mL	M3G	M6G	morphine	clonidine	MiOH	midazolam
0.05	65%	82%	38%	51%	67%	39%
0.1	88%	94%	75%	80%	36%	79%
0.5	92%	99%	68%	62%	33%	76%
1,00	83%	99%	78%	84%	36%	75%
50	87%	97%	69%	52%	35%	88%
2.5	58%	96%	88%	81%	36%	66%
5	77%	95%	37%	34%	-29%	79%
25	83%	95%	68%	26%	33%	82%
100	80%	96%	64%	64%	6%	68%
Average	**79%**	**95%**	**65%**	**59%**	**28%**	**72%**

Calibrator concentration, ng/mL	M3G-D3	M6G-D3	morphine-D6	clonidine-D4	MiOH-D4	midazolam-D6
0.05	78%	94%	35%	51%	19%	19%
0.1	79%	94%	69%	76%	22%	10%
0.5	89%	96%	67%	61%	32%	25%
1,00	82%	95%	78%	86%	36%	26%
50	86%	95%	69%	57%	38%	21%
2.5	64%	-32%	90%	83%	37%	37%
5	68%	91%	30%	39%	-36%	22%
25	84%	95%	84%	62%	31%	18%
100	80%	94%	74%	76%	39%	32%
Average	**79%**	**80%**	**66%**	**66%**	**24%**	**23%**

Annex 3. Comparison of carry-over for different methods.

Compound	Method A	Method A	Method B	Method C	Method D	Method E	Method F	Method F	Method G	Method G	Method H	Method H
	1st blank injection after 50 ng/mL sample	2nd blank injection after 50 ng/mL sample	1st blank injection after ULOQ sample	1st injection after ULOQ sample	1st injection after ULOQ sample	1st injection after ULOQ sample	1st blank injection after 50 ng/mL sample	2nd blank injection after 50 ng/mL sample	1st injection after ULOQ	2nd injection after ULOQ	1st injection after ULOQ	2nd injection after ULOQ
M3G	560%	494%	717%	0,6%	0%	0%	560%	494%	0%	42%	0%	0%
M3G-D_3	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
M6G	472%	227%	1103%	168%	0%	0%	472%	227%	0%	136%	0%	0%
M6G-D_3	1%	0%	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%
morphine	180%	38%	644%	347%	105%	56%	180%	38%	0%	87%	0%	0%
morphine-D_6	1%	0%	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%
clonidine	85%	12%	473%	109%	58%	0%	85%	12%	0%	0%	0%	0%
clonidine-D_4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
MiOH	140%	46%	588%	105%	149%	173%	140%	46%	29%	46%	86%	0%
MiOH-D_4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
midazolam	91%	7%	382%	18%	121%	134%	91%	7%	51%	34%	46%	0%
midazolam-D_6	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Annex 4. Precision and accuracy data for all compounds in LLOQ level for 1st and 2nd day.

Analyte; expected conc. ng/mL	Day 1					Day 2				
	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %
M3G; 0.055	0.053	0.053	0.002	97	3.1	0.054	0.054	0.002	99	4.3
	0.052					0.051				
	0.053					0.058				
	0.052					0.056				
	0.057					0.053				
M6G; 0.055	0.048	0.053	0.006	96	10.8	0.058	0.056	0.004	102	7.8
	0.048					0.056				
	0.063					0.049				
	0.055					0.057				
	0.050					0.062				
Morphine; 0.055	0.063	0.060	0.002	109	3.3	0.052	0.055	0.002	100	3.7
	0.060					0.058				
	0.059					0.055				
	0.058					0.056				
	0.059					0.054				
Clonidine; 0.052	0.054	0.052	0.002	100	4.3	0.058	0.059	0.002	113	2.6
	0.051					0.057				
	0.055					0.061				
	0.049					0.060				
	0.053					0.058				
MiOH; 0.052	0.058	0.055	0.003	102	4.8	0.056	0.056	0.002	103	3.3
	0.054					0.054				
	0.058					0.058				
	0.051					0.057				
	0.056					0.053				
Midazolam; 0.054	0.049	0.049	0.001	91	2.4	0.048	0.047	0.001	87	1.8
	0.049					0.046				
	0.052					0.048				
	0.048					0.046				
	0.050					0.046				

Precision and accuracy data for all compounds in LLOQ level for 3rd day.

Analyte; expected conc. ng/mL	Day 3				
	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %
M3G; 0.051	0.057	0.056	0.002	111	4.4
	0.058				
	0.059				
	0.054				
	0.053				
M6G; 0.050	0.048	0.050	0.005	99	10.2
	0.055				
	0.042				
	0.053				
	0.009*				
Morphine; 0.051	0.046	0.049	0.003	96	5.8
	0.050				
	0.048				
	0.047				
	0.054				
Clonidine; 0.048	0.056	0.053	0.002	111	4.1
	0.056				
	0.050				
	0.052				
	0.054				
MiOH; 0.050	0.0548	0.0539	0.0011	108	2.1
	0.0536				
	0.0519				
	0.0552				
	0.0540				
Midazolam; 0.050	0.053	0.050	0.002	101	3.7
	0.052				
	0.048				
	0.051				
	0.049				

*Result was excluded from calculations because it didn’t fit accuracy parameter

Annex_A Precision and accuracy data for M3G, M6G, morphine and clonidine in 3xLLOQ level for 1st and 2nd day.

Analyte; expected conc. ng/mL	Day 1					Day 2				
	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %
M3G; 0.159	0.148	0.149	0.005	94	3.5	0.159	0.155	0.006	97	3.7
	0.144					0.165				
	0.153					0.151				
	0.143					0.149				
	0.156					0.152				
						0.151				
M6G; 0.158	0.168	0.163	0.007	103	4.5	0.151	0.162	0.013	102	7.9
	0.167					0.168				
	0.164					0.140				
	0.167					0.179				
	0.149					0.166				
						0.168				
Morphine; 0.159	0.156	0.160	0.008	100	4.9	0.151	0.146	0.005	92	3.3
	0.159					0.144				
	0.171					0.139				
						0.142				
	0.148					0.152				
	0.164					0.147				
Clonidine; 0.158	0.155	0.153	0.002	97	1.3	0.144	0.149	0.004	94	2.9
	0.155					0.143				
	0.151					0.152				
						0.153				
	0.152					0.151				
	0.155					0.152				

Precision and accuracy data for MiOH and midazolam in 3xLLOQ level for 1st and 2nd day.

MiOH; 0. 158	0.160	0.158	0.005	101	3.2	0.199*	0.164	0.002	105	1.1
	0.153					0.164				
	0.163					0.166				
	0.152					0.164				
						0.161				
	0.164					0.166				
Midazolam; 0.054	0.137	0.137	0.002	87	1.1	0.137	0.141	0.003	90	2.1
	0.136					0.139				
	0.136					0.143				
	0.136					0.141				
						0.144				
	0.140					0.145				

Precision and accuracy data for all compounds in 3xLLOQ level for 3rd day.

Analyte; expected conc. ng/mL	Day 3				
	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc., %	CV, %
M3G; 0.145	0.124	0.136	0.008	94	5.7
	0.133				
	0.138				
	0.137				
	0.148				
M6G; 0.145	0.136	0.141	0.005	97	3.2
	0.141				
	0,041*				
	0.138				
	0.148				
Morphine; 0.146	0.167	0.160	0.004	109	2.6
	0.157				
	0.160				
	0.161				
	0.154				
Clonidine; 0.145	0.135	0.135	0.003	93	2.0
	0.137				
	0.136				
	0.130				
	0.136				
MiOH; 0.144	0.145	0.146	0.003	101	2.1
	0.144				
	0.146				
	0.142				
	0.151				
Midazolam; 0.145	0.128	0.130	0.002	90	1.2
	0.129				
	0.132				
	0.131				
	0.130				

*Result was excluded from calculations because it did not meet accuracy criteria

Precision and accuracy data for all compounds in MED level for 1st and 2nd day.

Analyte; expected conc. ng/mL	Day 1					Day 2				
	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %
M3G; 55.0	55.1	53.3	1.1	97	2.1	49	53	2	95	3.0
	51.7					53				
	52.7					54				
	53.3					54				
	53.6					53				
M6G; 55	59	50	4	91	8.9	48.5	49.2	1.3	90	2.7
	47					48.0				
	51					48.8				
	47					51.5				
	48					43,6*				
Morphine; 54.8	53.8	52.3	1.0	96	1.9	50.8	52.3	1.0	95	1.9
	52.5					52.2				
	50.6					52.9				
	52.3					51.7				
	52.6					53.8				
Clonidine; 55.2	52.8	51.7	0.6	94	1.1	49.7	51.9	1.2	94	2.3
	51.2					53.1				
	51.1					52.7				
	51.8					52.0				
	51.8					52.2				
MiOH; 54.9	59.8	58.4	0.7	106	1.2	55	58	2	105	2.9
	58.0					59				
	58.0					59				
	58.6					59				
	57.9					58				
Midazolam; 54.9	49.3	49.5	1.1	90	2.2	47.1	48.5	0.9	88	1.8
	48.2					49.0				
	49.1					49.6				
	49.4					47.9				
	51.5					48.6				

* Result was excluded from calculations because it did not meet accuracy criteria

Precision and accuracy data for all compounds in MED level for 3rd day.

Analyte; expected conc. ng/mL	Day 3				
	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %
M3G; 49.2	45.7	47.3	1.0	96	2.2
	47.1				
	46.9				
	48.7				
	48.1				
M6G; 49	44	45	2	92	5.0
	43				
	37*				
	44				
	49				
Morphine; 49	49	52	2	107	4.2
	51				
	53				
	54				
	55				
Clonidine; 49.4	49.0	48.0	0.9	97	2.0
	46.3				
	47.9				
	48.7				
	48.4				
MiOH; 49.1	52.5	52.1	1.4	106	2.6
	51.0				
	50.3				
	54.2				
	52.5				
Midazolam; 49.2	44.3	43.7	0.3	89	0.7
	43.3				
	43.5				
	43.6				
	43.8				

* Result was excluded from calculations because it did not meet accuracy criteria

Precision and accuracy data for all compounds in ULOQ level for 1st and 2nd day.

Analyte; expected conc. ng/mL	Day 1					Day 2				
	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %
M3G; 202	230	204	10	102	4.7	193	201	5	100	2.3
	201					204				
	200					202				
	201					202				
	199					206				
M6G; 214	185	189	10	90	5.1	179*	187	3	87	1.4
	186					183				
	198					187				
	201					187				
	198					191				
Morphine; 204	232	202	10	99	5.2	194	201	5	99	2.5
	197					200				
	200					202				
	194					200				
	190					210				
Clonidine; 198	259*	212	7	105	3.3	203	215	7	108	3.5
	210					224				
	211					217				
	214					210				
	198					220				
MiOH; 205	279*	226	6	111	2.8	215	226	6	110	2.7
	228					233				
	230					227				
	225					223				
	227					230				
Midazolam; 194	259*	215	8	110	3.5	208	217	5	112	2.2
	215					221				
	213					218				
	212					217				
	215					221				

Precision and accuracy data for all compounds in ULOQ level for 3rd day.

Analyte; expected conc. ng/mL	Day 3				
	Measured conc., ng/mL	Average, ng/mL	STD, ng/mL	Acc. , %	CV, %
M3G; 194	200	198	3	102	1.3%
	198				
	198				
	202				
	194				
M6G; 205	172	173	2	85	1.0%
	168*				
	173				
	176				
	168*				
Morphine; 195	213	210	4	108	1.8%
	211				
	214				
	203				
	209				
Clonidine; 190	207	208	3	110	1.6%
	208				
	211				
	212				
	203				
MiOH; 196	208	213	4	109	1.9%
	208				
	212				
	218				
	217				
Midazolam; 187	213	203	5	109	2.6%
	201				
	203				
	201				
	197				

* Result was excluded from calculations because it did not meet accuracy criteria

Annex 5. Recovery and process efficiency.

Recovery

Analyte	LLOQ		3xLLOQ		MED	
		Std, %		Std, %		Std, %
M3G	80%	5.7%	70%	3.9%	66%	1.1%
M6G	93%	6.3%	81%	12.8%	65%	3.4%
morphine	85%	8.3%	69%	2.2%	62%	4.9%
clonidine	86%	3.8%	71%	1.9%	62%	1.7%
MiOH	80%	2.3%	69%	2.8%	70%	2.1%
midazolam	79%	3.4%	70%	1.7%	65%	2.1%

Process efficiency

Analyte	LLOQ		3xLLOQ		MED	
	PE, %	Std, %	PE, %	Std, %	PE, %	Std, %
M3G	68%	5.6%	68%	3.6%	64%	1.7%
M6G	143%	11.9%	96%	9.5%	64%	3.0%
morphine	110%	13.3%	80%	4.4%	66%	3.4%
clonidine	82%	12.1%	77%	4.4%	69%	2.3%
MiOH	78%	4.6%	76%	3.7%	75%	1.9%
midazolam	78%	3.5%	74%	2.5%	68%	1.2%

Annex 6. Short term stability results.

Freeze and thaw stability

Analytes	LLOQ		3xLLOQ		MED	
	ST, %	Std, %	ST, %	Std, %	ST, %	Std, %
M3G	104	14	115	4	98	2
M6G	88	14	89	15	107	4
morphine	76	10	100	25	101	4
clonidine	98	3	105	11	101	2
MiOH	101	3	108	9	98	4
midazolam	103	6	108	9	100	4

Bench-top stability

Analytes	LLOQ		3xLLOQ		MED		ULOQ	
	ST, %	Std, %	ST, %	Std, %	ST, %	Std, %	ST, %	Std, %
M3G	92	5	101	4	102	3	102	2
M6G	91	15	97	8	100	4	103	2
morphine	85	11	86	4	89	4	89.2	1.4
clonidine	90	3	90	2	96.4	1.3	96.7	1.3
MiOH	103	3	98	2	98.7	1.2	104	4
midazolam	98	3	97	2	102	2	105	3

Sample stability in the autosampler

Analytes	LLOQ		3xLLOQ		MED		ULOQ	
	ST, %	Std, %	ST, %	Std, %	ST, %	Std, %	ST, %	Std, %
M3G	92	5	101	4	102	3	102	2
M6G	91	15	97	8	100	4	103	2
morphine	85	11	86	4	89	4	89.2	1.4
clonidine	90	3	90	2	96.4	1.3	96.7	1.3
MiOH	103	3	98	2	98.7	1.2	104	4
midazolam	98	3	97	2	102	2	105	3

Annex 7. Long term stability results.

Long term stability results, at LLOQ concentration level, sample at -80°C storage temperature

Time, months	M3G		M6G		morphine		clonidine		MiOH		midazolam	
	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %
1	99	6,9	84	1,4	96	23,3	81	1,5	91	2,1	81	1,2
2	88	9,7	82	4,5	80	16,3	98	2,2	92	1,5	79	1,0
3	97	4,1	76	1,6	75,0	0,5	92	1,6	101	1,1	87	2,0
4	101	1,3	77	10,4	70	12,6	66	5,3	94	2,2	79	0,4

Long term stability results, at LLOQ concentration level, sample at -20°C storage temperature

Time, months	M3G		M6G		morphine		clonidine		MiOH		midazolam	
	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %
1	75%	4,4%	82%	0,0%	93%	16,2%	84%	4,4%	89%	0,8%	82%	0,2%
2	85%	1,5%	80%	9,5%	78%	27,6%	94%	3,1%	97%	1,7%	84%	1,4%
3	90%	3,6%	76%	6,4%	109%	2,4%	93%	1,2%	101%	3,1%	89%	2,0%
4	104%	0,4%	70%	6,5%	71%	3,6%	92%	1,0%	104%	2,8%	92%	1,8%

Long term stability results, at ULOQ concentration level, sample at -80°C storage temperature

Time, months	M3G		M6G		morphine		clonidine		MiOH		midazolam	
	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %
1	98%	1,0%	95%	1,1%	101%	1,8%	103%	1,0%	104%	1,1%	100%	0,7%
2	97%	0,8%	85%	0,7%	109%	1,3%	102%	0,6%	103%	1,2%	96%	0,8%
3	100%	1,3%	95%	0,7%	102%	1,4%	105%	1,5%	103%	1,2%	101%	1,5%
4	108%	1,3%	109%	1,8%	108%	1,2%	112%	0,7%	111%	1,5%	111%	1,6%

Long term stability results, at ULOQ concentration level, sample at -20°C storage temperature

Time, months	M3G		M6G		morphine		clonidine		MiOH		midazolam	
	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %	Average, %	Std, %
1	99	0.3	97	0.4	109	0.9	107	0.6	107	0.7	101	0.7
2	100	0.3	85	0.7	111	3.0	107	1.0	108	0.5	105	0.4
3	107	0.6	98	0.9	106	1.6	113	1.9	111	0.8	106	1.8
4	102	0.9	98	0.4	102	1.1	107	1.1	105	0.9	102	1.9

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Sheet of information

Sheet of information for Rūta Veigure's Master's thesis (2016) "Development and validation of UHPLC-MS/MS method for analysis of sedative drugs and their metabolites in blood plasma"

Infoleht*. Rūta Veigure, magistritöö (2016) „UHPLC-MS/MS metoodika arendamine ja valideerimine uinutite ning nende metaboliitide määramiseks inimese vereplasmast."*

Keywords: UHPLC-MS/MS, blood plasma, sedative drugs, metabolites, morphine, morphine-3-β-glucuronide, morphine-6-β-glucuronide, clonidine, midazolam, 1'-hydroximidazolam

Märksõnad: *UHPLC-MS/MS, vereplasma, uinitid, metaboliidid, morfiin, morfiini-3-glükoroniid, morfiini-6-glükoroniid, klonidiin, midasolaam, 1'-hüdroksümidasolaam*

Abstract: In intensive care units, the precise administration of sedative drugs is crucial in order to avoid under- or over sedation – both of which can be very harmful. It is especially important in case of paediatric patients. A highly sensitive simultaneous UHPLC-MS/MS method was developed for the quantification of morphine, morphine-3-β-glucuronide, morphine-6-β-glucuronide, clonidine, midazolam and 1'-hydroximidazolam in human plasma samples. The lowest limit of quantification for all analytes was 50 pg/mL using only 100 µL of blood plasma. Analytes were separated chromatographically using C18 column with weak ion-pairing additive 1,1,1,3,3,3-hexafluoro-2-propanol with pH = 9 (adjusted using ammonium hydroxide) and methanol. Method was fully validated and method was using matrix matched calibration in the range of 0.05-250 ng/mL for all analytes. Within-day accuracy for all analytes remained 87-113 %, but within-day precision remained within 3-11 % for all analytes at all concentration levels over the calibration range. The method will be applied for real patients' samples in the EU FP7 project CloSed – "Clonidine for Sedation of Paediatric Patients in the Intensive Care Unit".

Lühikkokuvõte: *Õige koguse uinuti manustamine intensiivraviosakonnas on määrava tähtsusega. Mitte ainuüksi üle- vaid ka alamanustamine võib olla patsiendile äärmiselt kahjulik. Seda eriti juhul kui patsientideks on lapsed. Käesoleva töö käigus töötati välja metoodika mõnede uinutite – klonidiin, morfiin ja midasolaam ning nende metaboliitide (morfiini-3-glükoroniid, morfiini-6-glükoroniid 1'-hüdroksümidasolaam) samaaegseks määramiseks inimese vereplasmast, kasutades ülikõrgefektiivset vedelikkromatograafiat koos elektropihustusionisatsioon massispektromeetriga. Kõigi analüütide määramispiiriks sealjuures oli 50 pg/mL ning kasutatud proovi kogus kõigest 100 µL. Kromatograafiliste parameetrite optimeerimisel saavutati ainete lahutus gradientelueerimisel mobiilfaasiga, mis koosnes nõrgast ioon-paar reagendist 1,1,1,3,3,3-heksafluoro-iso-propanool (pH 9) ja metanoolist. Metoodika täielikul valideerimisel hinnati lineaarset ala läbi maatriksvastava kalibreerimise vahemikus 0.05-250 ng/mL kõikide analüütide jaoks. Kõikide analüütide päevasisene mõõtetäpsus jäi vahemikku 87-113 % ja kordustäpsus vahemikku 3-11 %. Metoodikat rakendatakse EU FP7 projekti CloSed – „Klonidiin sedatsiooniks lasteintensiivravi osakonnas" raames uinutite kontsentratsiooni määramiseks patsientide proovidest.*