A Highly Sensitive Method for the Simultaneous UHPLC-MS/MS Analysis of Clonidine, Morphine, Midazolam and their Metabolites in Blood Plasma Using HFIP as the Eluent Additive

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Abstract

In intensive care units, the precise administration of sedatives and analgesics is crucial in order to avoid under- or over sedation and for appropriate pain control. Both can be harmful to the patient, causing side effects or pain and suffering. This is especially important in the case of pediatric patients, and dose-response relationships require studies using pharmacokinetic-pharmacodynamic modeling. The aim of this work was to develop and validate a rapid ultra-high performance liquid chromatographic-tandem mass spectrometric method for the analysis of three common sedative and analgesic agents: morphine, clonidine and midazolam, and their metabolites (morphine-3-glucuronide, morphine-6-glucuronide and 1'-hydroxymidazolam) in blood plasma at trace level concentrations. Low concentrations and low sampling volumes may be expected in pediatric patients; we report the lowest limit of quantification for all analytes as 0.05 ng/mL using

only 100 µL of blood plasma. The analytes were separated chromatographically using the C18 column with the weak ion-pairing additive 1,1,1,3,3,3-hexafluoro-2-propanol and methanol. The method was fully validated and a matrix matched calibration range of 0.05 – 250 ng/mL was attained for all analytes In addition, between-day accuracy for all analytes remained within 93 – 108 %, and precision remained within 1.5 – 9.6 % for all analytes at all concentration levels over the calibration range.

Keywords

UHPLC-MS/MS, chromatographic separation, morphine, clonidine, midazolam, hexafluoroisopropanol.

1. Introduction

Sedation is commonly used in intensive care units (ICU), and there is an increasing recognition of the need to avoid over-sedation, study non-benzodiazepines (which may lead to withdrawal and tolerance) and ensure adequate analgesia according to individual needs [1]. Sedative and analgesic requirements of children admitted to neonatal or pediatric ICU are under-studied, meaning optimal dosing is unclear. Furthermore, the desire to avoid the potentially harmful effects of benzodiazepines by moving towards using alpha-2 adrenergic receptor agonists such as clonidine [2] are hampered by a lack of data on efficacy, safety and pharmacokinetics in the pediatric population.

The CLON01 study (EudraCT 2014-003582-24) "Clonidine for Sedation of Pediatric Patients in the Intensive Care Unit" (CloSed study) is a multicenter double-blind, randomized, controlled trial funded by the European Commission Framework 7 program comparing clonidine with midazolam used for sedation in neonatal and pediatric ICU (the current standard of care). In addition to these sedative drugs patients in either arm of this study do also receive morphine as analgesic component. A secondary endpoint of the study is to collect and analyze pharmacokinetic samples for all three substances to support the development of dose guidelines for sedation in neonatal and pediatric ICU.

Clonidine stimulates alpha (2)-adrenoceptors in the central nervous system which results in lowering blood pressure and decreasing of heart rate [3]. Because of that clonidine is used as an antihypertensive drug, but it is also used for multiple other indications such as sedation and analgesia [4].

Midazolam is a short-acting benzodiazepine with hypnotic, anticonvulsant, sedative, muscle-relaxant and anxiety preventing properties [5]. Midazolam is hydroxylated to its primary active metabolite – 1'-hydroxymidazolam (MiOH) [6], meaning quantification of both parent and metabolite will be important to investigate sedative activity and potential developmental differences in metabolic activity with age.

Morphine is a highly addictive analgesic [7]. About 56 % of the morphine is metabolized to morphine-3-glucuronide (M3G), and about 10 % to morphine-6-glucuronide (M6G) [8]. Both glucuronides are very hydrophilic, but M6G crosses the blood-brain barrier more readily and due to its different plasma-concentration profile as well as long brain extracellular fluid half-life, has

been found a more potent analgesic than M3G or even morphine [9]. As with midazolam, quantification of these metabolites will also be important.

The simultaneous quantitation of sedatives and analgesics and their active metabolites will allow complex evaluation of the pharmacokinetic/pharmacodynamic relationships and defining optimal dosing for sedation at the same time limiting sample volumes and resource needs.

In total, 10 liquid chromatography-mass spectrometry (LC-MS) methods were reviewed. The lowest limits of quantification (LLOQ) ranging from 2 – 9 ng/mL for morphine and its two major metabolites [7,10,11], 0.01 – 100 ng/mL for clonidine – but clonidine was the only single compound analyzed in these assays [3,4,12,13], 0.025 – 5 ng/mL for midazolam [10,11,14,15] and 0.1 – 2.5 ng/mL for MiOH [10,15]. The exception was the LLOQ of 50 fg/mL for midazolam and 0.25 pg/mL for MiOH [16].

Basic conditions for reversed phase (RP) separation are useful in the case of pharmaceutical analyte analyses since over 70 % of them have basic properties, but only approximately 20 % are acids [17]. Basic analytes are protonated if the eluent's pH is lower than the analyte's pK_a value and thus have poor retention in RP conditions [17]. Fluoroalcohols like 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, pK_a = 9.3 [18]) can be used in liquid chromatography - electrospray ionization source - mass spectrometry (LC-ESI-MS) as a buffer additive for basic solutions. Moreover, HFIP alters the selectivity of chromatographic separation. Fluoroalcohols are strongly retained on the hydrophobic RP stationary phase and thus create a hydrophilic layer with hydrogen bond donor properties. Furthermore, in the mobile phase the anions of fluoroalcohols form ion pairs with protonated bases and thus enhance their retention on the already altered stationary phase. Acidic compounds, however, have to compete with fluoroalcohols on the stationary phase surface, which decreases their retention [19].

We aimed to develop a method suitable for quantifying low levels of sedatives and their metabolites in the limited sample volume conditions – from neonatal and pediatric patients' blood plasma samples. Sufficiently low LLOQ levels will be necessary for obtaining adequate pharmacokinetic data for the evaluation of optimal dosing in the future.

2. Materials and methods

2.1. Chemicals

Standard substances and their respective stable isotope labeled 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 Cerilliant (Texas, USA). Clonidine-D_4 and midazolam-D_6 were obtained from the Toronto Research Chemicals Inc. (Toronto, Canada). Other reagents used: LC-MS Ultra chromasolv grade methanol (MeOH) from Sigma Aldrich (Missouri, USA), LC-MS grade formic acid from Sigma Aldrich (Missouri, USA), LC-MS grade ammonium hydroxide solution from Sigma Aldrich (Missouri, USA), LC-MS grade 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) from Sigma Aldrich (Missouri, USA). Water was purified (18.2 MΩ·cm at 25 °C and and a total organic carbon (TOC) value 2 – 3 ppb) in-house using a Millipore Advantage A10 system from Millipore (Bedford, USA). Plasma and whole blood were purchased from Blood Bank of Tartu University Hospital.

2.2. Sample preparation

Protein precipitation was accomplished by adding 50 µL methanol containing 10 ng/mL IS and 700 µL of neat methanol to 100 µL of each calibrator, quality control (QC) or sample. The resulting solution was mixed for 4 min in the Eppendorf MixMate mixer (Hamburg, Germany) and centrifuged at 30,000 × g for 10 min at 4 °C in the Eppendorf Centrifuge 5430 R (Hamburg, Germany). The supernatant (approximately 850 µL) was transferred into a 2 mL Eppendorf polypropylene vial and evaporated to dryness using the Jouan RC 10-09 centrifugal evaporator (Saint-Herblain, France) at 8 – 10 mbar pressure at 1200 rpm. Samples were reconstituted in 80 µL of water and methanol mixture (8:2, v/v). An aliquot of 6 µL was injected into the UHPLC-MS/MS system.

2.3. Chromatographic conditions

The Agilent 1290 Infinity (Santa Clara, USA) UHPLC system consisted of a binary pump, a thermostated column compartment and an autosampler (set at 4 °C). Analytes were separated using the Waters Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 µm) analytical column with the Waters VanGuard BEH C18 (2.1 × 5 mm, 1.7 µm) pre-column (Milford, USA), which were maintained at 30 °C. To protect both column and pre-column from unnecessary blockages, an in-line filter was installed ahead of them. The mobile phase consisted of water (solvent A) containing 5 mM HFIP (v/v) (at pH 9, adjusted with ammonium hydroxide solution) and methanol (solvent B).

The flow rate for the gradient elution was 250 µL/min. The gradient started from 5 % solvent B for the first minute, then was increased to 75 % until the 3.7 min and kept at 75 % until the 5.8 minute. Between the 5.8 and 5.9 min, the MeOH content was increased to 100 % and kept there until the 6.9 min, after which it was decreased back to 5 % in 0.1 min and kept at 5 % for 3 min to allow the column to equilibrate.

2.4. Mass spectrometry

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

The following mass analyzer settings were used: drying gas temperature 135 °C, drying gas flow rate 13 L/min, nebulizer pressure 25 psi (172 MPa), sheath gas temperature 400 °C and sheath gas flow (11 L/min), capillary voltage (2,500 V) and nozzle voltage (500 V). iFunnel voltage in the high pressure region was 210 V and at low pressure it was 220 V. Optimized collision energies for each analyte and the internal standard transitions are listed in Table 1. Fragmentation patterns for these analytes have been documented previously [13,16,20] and support the chosen *m/z* values for the method.

*****Table 1 goes here*****

2.5. Validation

The method was fully validated according to the European Medicines Agency (EMA) guideline [21]. The linear range, the LLOQ, the method's within-day and between-day accuracy and precision were evaluated. Moreover, an estimation of the matrix effect, carry-over, selectivity and analyte stability under different storage conditions was conducted.

In order to evaluate selectivity, independent blank plasma samples were analyzed. The analysis of the double blank plasma sample (blank plasma without the addition of an internal standard during sample preparation) was conducted every time when calibration samples were analyzed in order to re-assure selectivity.

The carry-over was evaluated by comparing the peak areas in the blank sample injected after the higher concentration sample with the peak areas of the LLOQ sample.

Accuracy and precision were evaluated with QC samples at four concentration levels by analyzing 5 independently prepared samples at each level in every analytical run. QC samples were spiked using separate standard solutions with appropriate dilutions which were quantified using the calibration samples. According to the EMA guideline [21] for accuracy, the measured concentration should be within 15 % of the nominal value (20 % at the LLOQ concentration level). The criterion for the precision is 15 % (20 % at the LLOQ level).

Matrix effects (MEs) were estimated according to the EMA guideline [21] and as described by Matuszewski et al. [22]. The ME was evaluated as the ratio of the signal from the post-extraction spiked sample to the standard solution (at the same concentration level). For the ME evaluation, the analytes in neat solvent (standard solution) and the post-extraction spiked samples were analyzed. MEs in MS detection occur due to the components of the sample matrix which have not been removed during sample preparation. In the case of blood plasma samples, the ME is caused mostly by phospholipids that either enhance (ME over 100 %) or suppress (ME under 100 %) the analytical signal. In addition, the interference in assay performance while using hemolyzed and hyperlipidemic plasma was evaluated.

Freeze and thaw stability was assessed in order to take into account the possible degradation in the case of the accidental thawing of samples during transportation, but also the possible bias caused by degradation during the incurred sample reanalysis. In order to evaluate this, spiked plasma samples at three concentrations - LLOQ, three times lowest limit of quantification (3xLLOQ) and medium concentration (MED) were frozen in a freezer at -80 ºC and thawed at room temperature in three cycles. In the course of each cycle, the samples were kept at -80 ºC for at least 24 h.

For the evaluation of bench-top stability, spiked plasma samples at four concentrations - LLOQ, 3xLLOQ, MED and upper limit of quantification (ULOQ), were kept at monitored room temperature (22 ºC ± 1 ºC) for 24 h.

For the evaluation of 24-hour stability, the samples were kept in an autosampler at an average temperature of 4 ºC.

In addition, long term (9 months) stability tests were conducted, keeping low (LLOQ 0.05 ng/mL) and high (ULOQ 200 ng/mL) concentration samples in a freezer at -20 ºC and -80 ºC in order to evaluate the most suitable storage conditions.

3. Results and discussion

For the method development and validation, no clinical study samples were analyzed.

3.1. Method development and the chromatographic retention mechanism of the analytes

*****Figure 1 goes here*****

The analytes analyzed have basic properties - morphine's pK_a = 8.2 [23], clonidine's pK_a = 8.05 [24], MiOH's pK_a is estimated to be 4.99 [25], midazolam's pK_a = 6.15 [26] and the eluent's water phase additive HFIP is a weak acid (pK_a = 9.3 [18]), thus the eluent's pH was adjusted to pH = 9 using ammonium hydroxide. Glucuronides' pK_a values should be similar to those of morphine, however they also have a carboxylic acid group with the pK_a value ranging 3-4 [27].

HFIP was added to enhance the compounds' signal in mass spectrometric detection [28] and to allow alternative chromatographic separation for morphine, M3G and M6G. The two metabolites share the same MS transitions (Table 1) and must therefore be chromatographically separated.

For simultaneous analysis of morphine and its glucuronides, they must be chromatographically separated. Namely, M3G and M6G partly undergo in-source collision induced dissociation (CID) losing the glucuronide moiety, which leads to *m/z* identical to that of morphine (*m/z* 286). Without chromatographic separation, glucuronides would contribute to the signal of morphine [20].HFIP acts as a weak ion-pairing additive in the basic mobile phase and therefore provides alternative selectivity in the C18 stationary phase [19]. Due to the carboxylic acid group in M3G and M6G, it is completely deprotonated at the used eluent pH 9.0. As a result, analytes are ionized and therefore elute early from the chromatographic system. Alternative selectivity compared to the commonly used C18 stationary phase is provided by HFIP, which interacts strongly with RP and forms a fluorous layer on its surface.

Morphine and clonidine have similar pK_a values, thus their complete chromatographic separation was challenging. Both compounds' pK_a values are lower than the pH of the eluent and being basic compounds they are deprotonated (neutral), explaining longer retention times than those of glucuronides. The fluorinated stationary phase provides alternate selectivity for the

fluorinated analytes [29] midazolam and MiOH. Besides having a similar structure to midazolam, MiOH is more polar and it elutes faster.

3.2. Method validation

3.2.1. Selectivity

Selectivity was evaluated by analyzing 6 independent blank plasma samples. None of the analyzed plasma samples contained peaks on method transitions and retention times (Table 1).

3.2.2. Carry-over

During the method development, carry-over was observed. Extended needle and seal wash programs and multiple blank injections with prolonged washing with 100 % MeOH did not decrease the carry-over sufficiently. Injecting 0.1 % formic acid solution decreased carry-over significantly, but not sufficiently, thus it was decided to completely change the eluent A to 0.1 % formic acid solution for the wash program after the injection of high concentration samples. The acidic eluent was chosen to improve the solubility of basic analytes. In acidic conditions, basic analytes become protonated, thus they become polar and less retained on the non-polar C18 stationary phase or on the needle and seal. In order to further clean the system, the needle wash with MeOH and 0.1 % formic acid 1:1 (v/v) was conducted. The wash program with 0.1 % formic acid as the eluent A proved to be more efficient compared to the basic eluent. Due to the low concentration of IS, no carry-over was observed for them. The conducted clean-up gradient with 0.1 % formic acid and MeOH helped to remove the carry-over for all analytes.

3.2.3. The lower limit of quantification

The LLOQ's achieved are listed in Table 2. The target LLOQ levels were achieved with the signal to noise ratio (s/n) 5 or higher (as determined by the Agilent MassHunter Qualitative Analysis software version B.06.00) and the LLOQ level accuracy and precision were within 20 % – as required by the EMA guideline [21]. The LLOQ was additionally assessed with regard to accuracy and precision.

3.2.4. Calibration curve

The matrix matched calibration consisted of 10 concentration levels in addition to the blank and double blank samples and was analyzed in duplicates. The range 0.05 – 250 ng/mL was chosen to fit the expected concentrations in the CloSed clinical trial samples based on the previous studies and the aim of the clinical trial to lower the concentration of sedatives. The curve was constructed using linear regression with $1/x^2$ (for M6G, clonidine, MiOH and midazolam) and 1/x (for M3G and morphine) weighting, the squared regression coefficient for all analytes was > 0.9930 (Table 2). All calibration curve points were within the set accuracy limits of 85 – 115 % [21] of their back-calculated values.

3.2.5. Accuracy

Accuracy is influenced by both the analyte and the concentration level at low concentrations when the noise level is more influential and peak shapes are often not ideal, while the range of the accuracy is wider. This is especially true for within-day accuracies at the LLOQ level ranging from 97 % to 111 % for M3G, from 100 % to 113 % for clonidine and from 87 % to 101 % for midazolam. Morphine's within-day accuracies varied in the range of 96 – 109 % for the LLOQ QC level. Accuracies for M6G and MiOH ranged from 96 % to 102 % and from 102 % to 108 %, respectively, at the LLOQ level. Within-day accuracies at all other concentration levels for all analytes ranged from 87 % to 110 %, except for M6G ranging from 85 % to 103 %. In general, at the ULOQ concentration level, the QCs were slightly overestimated, except for M6G. The biggest variations in the M6G accuracies could have been caused by the non-ideal peak shape in the chromatograms. Between-day accuracies are presented in Table 2.

*****Table 2 goes here*****

3.2.6. Precision

Precision is expressed as the coefficient of variation (CV) [21]. Similarly to accuracy, also precision (between-day precision is presented in Table 2) depended on the analyte and concentration level, while low concentrations are more influenced and show higher variation. In this regard, especially M6G stands out with the CV being 9.6 % at the LLOQ level, which is also the largest among all the analytes at all concentration levels. Precision is affected by high noise levels at low concentrations of glucuronides. The variability of the within-day precision was larger,

however trends remained the same – lower concentrations were affected more. The imprecision was highest for M6G at LLOQ concentration level: between 7.8 – 10 %. The smallest within-day variation at low concentration was observed for midazolam, ranging between 1.1 – 2.1 %. However, precision values (both within-day and between-day) remained within the allowed range, according to the guideline [21] for all of the compounds – below 15 % for the 3xLLOQ, MED and ULOQ levels and below 20 % for the LLOQ level.

3.2.7. Matrix effect

A stronger ME was observed at low concentration levels. M6G was influenced the most by the signal enhancement (ME 125 % at the LLOQ level). The ME for morphine also indicated a signal enhancement at the LLOQ level with the ME of 117 %. For other compounds, signal suppression was observed in the range of 83 – 98 %. For all compounds at the 3xLLOQ level, the ME ranged from 106 – 118 % except M3G which was at 98 %. At the MED level, the ME ranged from 95 – 109 % for all compounds (see supplementary data). The use of matrix matched calibration and the IS enabled the MEs to be accounted for.

In addition, hemolyzed and hyperlipidemic plasmas were tested (see supplementary data) in order to evaluate matrix effects. Hemolyzed plasma samples were prepared by spiking pure plasma with whole blood in the 0 – 10 % range of whole blood content in the plasma. All analytes' concentrations were within 85 – 104 % when compared to pure plasma samples at a low (at the LLOQ level, 0.05 ng/mL) concentration. Results were not influenced by hemolysis at higher concentrations, ranging from 87 % to 103 %. However, the influence of hemolysis was increased at a high concentration (at the ULOQ level, 200 ng/mL) for MiOH when the whole blood content in the plasma sample was 5 % and 7 % (difference from the non-hemolyzed plasma was 19 – 20%). The higher hemolysis rate is influencing the most determination of MiOH at the high concentration level and should be encountered while analyzing hemolyzed plasma. Hyperlipidemia did not influence accuracies strongly, the change was 87 – 103 % for LLOQ samples and 90 – 105 % for ULOQ samples.

3.2.8. Stability (ST)

Substock stability. Stability of substocks (in water) were tested comparing freshly made substocks (in the concentration range 0.9 ng/mL – 5 µg/mL), and substocks which were kept in a

freezer at -80 ºC for 8 months. Only minimal differences in concentrations were observed. Both glucuronides and morphine remained 100 – 103 % from their original concentrations and experienced no degradation over 8 months at -80 ºC (with the standard deviation of 4 – 6 % over all compared concentrations). Clonidine, midazolam and MiOH retained 94 – 99 % of the original content.

Freeze and thaw stability. Analytes at low concentrations were affected by repeated freezing and thawing the most, but virtually no influence was evident when the analytes' concentration was higher than 50 ng/mL (Figure 2). The biggest degradation occurred at the LLOQ level for morphine (only 76 % of the initial concentration remained after three thawing cycles), and for M6G with 88 % of its initial concentration.

*****Figure 2 goes here*****

Short term stability or bench-top stability. Variations for all analytes at all four concentration levels were within 85 – 105 % (see supplementary data), except for morphine at the LLOQ and 3xLLOQ concentration levels. Morphine's concentration after 24 h was 69 – 84 % at low concentrations levels, but the standard deviation for these results was high. Based on the short term stability results, it was decided to decrease the period for which the samples were kept at room temperature to a minimum (up to 60 minutes as absolute maximum for clinical trial samples).

24 h stability in the autosampler at 4 °C. Concentration variations were within 85 – 105 % for all compounds at all four concentration levels (Table 3).

*****Table 3 goes here*****

Long term stability at -20 ºC and -80 ºC.

Samples at the LLOQ concentration level (Figure 3) were stable for 9 months (85 – 110 %) when kept in a freezer at -80 ºC, which is in the range of permitted difference (15 %). Storage at -20 ºC showed a bigger decrease (82 – 103 %) in the compounds' concentrations and larger variation in results. Previously published studies show that analytes were stable both in long and short term even if kept at -20 ºC for different periods of time, [3,7,30]; however, the published lower concentrations were significantly higher compared to our assay. Only a minimal decrease in

concentrations was observed – for M3G and M6G by 7 % and for morphine by 5 % after 6 months of storage [7]. Clonidine was stable for 4 months – a decrease by 11 % in low concentrations and by 2.6 % in high concentrations was observed [3]. Midazolam and MiOH were stable in spiked plasma samples for at least 10 months [30], however no data were shown to support this claim.

*****Figure 3 goes here*****

Samples at the ULOQ concentration level (Figure 4) were not strongly influenced by different temperatures: 99 – 106 % when kept in a freezer at -80 ºC and 97 – 106 % when samples were kept in a freezer at -20 ºC. Based on the results from the LLOQ samples, we recommend strongly for the future studies to store all the study samples at -80 ºC over the longer periods (such as 9 months) of time.

*****Figure 4 goes here*****

4. Conclusions

A highly sensitive simultaneous UHPLC-MS/MS method was developed for the simultaneous quantification of morphine, morphine-3-β-glucuronide, morphine-6-β-glucuronide, clonidine, midazolam and 1'-hydroximidazolam in human plasma samples. The use of HFIP as an eluent additive improved the chromatographic separation of basic and zwitterionic compounds (especially between structurally similar morphine glucuronides) and increased the signal intensity of analytes, helping to achieve required LLOQ levels of 0.05 ng/mL using only 100 µL of blood plasma. Matrix effects were assessed for all compounds and were compensated with the usage of matrix matched calibration and stable isotope labeled internal standards for every analyte. It is essential to assess compound stability with subsequent consideration during different steps of clinical research, such as sampling, sample storage and transportation, as well as interpretation of results in the evaluation of pharmacokinetics.

The assay will be used to analyze pediatric patients' samples in the EU FP7 project CloSed – "Clonidine for Sedation of Pediatric Patients in the Intensive Care Unit".

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Conflict of Interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript, apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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Table 1. Retention times, monitored transitions and respective collision energies (CE) for analytes and IS-s.

Analyte	Retention time, min	*m/z**	Quantifier, *m/z*	Quantifier CE, V	Qualifier, *m/z*	Qualifier CE, V	References for transitions
M3G	3.50	462.2	286.0 $[M + H - C_6H_9O_6]^+$	26	200.8	46	[22]
M3G-D_3	3.50	465.2	289.0	32	-		
M6G	3.95	462.2	286.0 $[M + H – C_6H_9O_6]^+$	32	200.8	52	[22]
M6G-D_3	3.95	465.2	289.1	36	-		
morphine	4.80	286.2	152.0	64	165.1 $[C_8H_{11}N]^+$	49	[22]
morphine-D_6	4.80	292.2	152.0	64	-		
clonidine	4.91	230.0	44.0 $[C_2H_6N]^+$	29	212.9	28	[13]
clonidine-D_4	4.91	234.1	48.2	32	-		
MiOH	5.70	342.1	203.0 $[C_{10}H_6ClN_3]^+$	29	324.0	21	[18]
MiOH-D_4	5.70	346.1	202.9	29	-		
midazolam	6.06	326.1	291.1 $[M + H – Cl]^+$	29	222.0	57	[18]
midazolam-D_6	6.06	332.1	297.0	32	-		

*All precursor ions were [M + H]

Table 2. Validation parameters of the quantitative performance of the used sample preparation and detection method for all analytes.

Analyte	Linearity R^2	Weighting	LLOQ			3xLLOQ			MED			ULOQ		
			Mean calc. conc ng/mL	Accuracy, %	Precision, CV [%]	Mean calc. conc ng/mL	Accuracy, %	Precision, CV [%]	Mean calc. conc ng/mL	Accuracy, %	Precision CV [%]	Mean calc. conc ng/mL	Accuracy, %	Precision CV [%]
M3G	0.9992	1/x	0.055	102	3.9	0.147	95	4.3	51	96	2.4	201	101	2.8
M6G	0.9935	$1/x^2$	0.053	99	9.6	0.155	101	5.2	48	91	5.5	183	87	2.5
morphine	0.9989	1/x	0.055	102	4.3	0.155	101	3.6	52	99	2.7	205	102	3.2
clonidine	0.9969	$1/x^2$	0.055	108	3.7	0.146	95	2.1	51	95	1.8	212	108	2.8
MiOH	0.9939	$1/x^2$	0.055	104	3.4	0.156	102	2.1	56	106	2.2	222	110	2.7
midazolam	0.9930	$1/x^2$	0.049	93	2.6	0.136	89	1.5	47	89	1.6	212	110	2.8

Table 3. Results for 24 h stability (ST%) in the autosampler at 4 °C and at four concentration levels with standard deviation (SD).

Analytes	LLOQ		3xLLOQ		MED		ULOQ	
	ST%	SD, %	ST%	SD, %	ST%	SD, %	ST%	SD, %
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

Fig. 1 A typical chromatogram obtained at analytes' concentration of 0.5 ng/mL. (1 - M3G, 2 - M6G, 3 - morphine, 4 - clonidine, 5 - MiOH, 6 - midazolam)

Fig. 2 Freeze and thaw stability for all analytes at three concentration levels (LLOQ - 50 pg/mL, 3xLLOQ - 150 pg/mL and MED - 50 ng/mL) with standard deviation

Fig. 3 Long term stability with standard deviation for all analytes at the LLOQ concentration level after 9 months

Fig. 4 Long term stability with standard deviation for all analytes at the ULOQ concentration level after 8 months