Journal of Chromatography B, 1052 (2017) 150–157

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

Rūta Veigure[a], Rudolf Aro[a], Tuuli Metsvaht[b], Joseph F. Standing[c,d], Irja Lutsar[e], Koit Herodes[a], Karin Kipper[a,d,*], on behalf of the CloSed Consortium[1]

[a] University of Tartu, Institute of Chemistry, 14a Ravila Street, 50411 Tartu, Estonia
[b] Tartu University Hospital, Lunini 6, 51014 Tartu Estonia
[c] Inflammation, Infection and Rheumatology Section, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, United Kingdom
[d] Paediatric Infectious Diseases Research Group, Institute for Infection and Immunity, St. George's, University of London, Cranmer Terrace, London, SW17 0RE, United Kingdom
[e] University of Tartu, Institute of Microbiology, 19 Ravila Street, 50411 Tartu, Estonia

ARTICLE INFO

Article history:
Received 19 January 2017
Received in revised form 1 March 2017
Accepted 9 March 2017
Available online 10 March 2017

Keywords:
UHPLC–MS/MS
Chromatographic separation
Morphine
Clonidine
Midazolam
Hexafluoroisopropanol

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.

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.

* Corresponding author at: University of Tartu, Institute of Chemistry, 14a Ravila Street, 50411, Tartu, Estonia.
E-mail address: karin.kipper@gmail.com (K. Kipper).
[1] See supplementary file on doi: 10.1016/j.jchromb.2017.03.007.

http://dx.doi.org/10.1016/j.jchromb.2017.03.007
1570-0232/

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 to 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 p K_a value and thus have poor retention in RP conditions [17]. Fluoroalcohols like 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, p K_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-D3, M6G-D3, morphine-D6 and MiOH-D4 were obtained from Cerilliant (Texas, USA). Clonidine-D4 and midazolam-D6 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 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 min. 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 (2500 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.