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Validation of a HPLC/MS Method for Simultaneous Quantification of Clonidine, Morphine and its Metabolites in Human Plasma

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Abstract

A high-performance liquid chromatography-tandem mass spectrometry method was developed and validated for the simultaneous quantification of morphine, morphine's major metabolites morphine-3-glucuronide and morphine-6-glucuronide, and clonidine, to support the pharmacokinetic analysis of an ongoing double-blinded randomized clinical trial that compares the use of morphine and clonidine in infants diagnosed with neonatal abstinence syndrome. Plasma samples were processed by solid phase extraction and separated on an Inertsil ODS-3 (4- μ m) column using an 0.1% formic acid in water/0.1% formic acid in methanol gradient. Detection of the analytes was conducted in the positive multiple reaction monitoring mode. The range of quantitation was 1–1000 ng/mL for morphine, M3G and M6G, and 0.25–100 ng/mL for clonidine. Intraday and interday accuracy and precision were $\pm$ 15% for all analytes across the quantitation range. Extraction recovery rates were 94% for morphine, 90% for M3G, 87% for M6G, and 79% for clonidine. Matrix effect ranged from 85%–94% for clonidine to 101–106% for M3G. The method fulfilled all predetermined acceptance criteria and required only 100 μ L of starting plasma volume. Furthermore, it was successfully applied to 30 clinical trial plasma samples.

Keywords

LC-MS/MS; neonatal abstinence syndrome; morphine; clonidine; solid phase extraction; method validation

1. Introduction

Neonatal abstinence syndrome (NAS) is a withdrawal syndrome in infants with in utero exposure to opioids. NAS has become a serious public health issue in recent years, as its incidence and associated health expenditure have increased significantly in the past decade (Patrick, Davis, Lehmann, & Cooper, 2015; Patrick et al., 2012). Pharmacologic therapy is

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indicated for treating moderate to severe symptoms in NAS if patients fail to respond to nonpharmacologic therapy. The American Academy of Pediatrics Guidelines recommended the use of oral morphine and methadone as first-line agents (Hudak & Tan, 2012). However, the optimal initial drug therapy is still unknown, and there are ongoing clinical studies that are intended to address this issue (Reddy, Davis, Ren, & Greene, 2017).

Morphine is a μ -opioid agonist for treatment of moderate to severe pain. It is metabolized extensively through glucuronidation by UDP-glucuronosyltransferase-2B7 (UGT2B7) to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (Coffman, Rios, King, & Tephly, 1997). M6G has been shown to have strong binding affinity for the μ -opioid receptor and have significant contribution to analgesia produced by morphine. M3G, on the other hand, has no analgesic properties due to its low affinity for opioid receptors (Christrup, 1997). M3G has been associated with some neurotoxic symptoms, including hyperalgesia and allodynia (Andersen, Christrup, & Sjogren, 2003).

The use of clonidine as an adjunctive therapy in NAS has been investigated in several studies (Agthe et al., 2009; Surran et al., 2013). Its use as the primary treatment was first reported in several case series (Esmaeili et al., 2010; Hoder et al., 1984; Leikin et al., 2009; O'Mara, Gal, & Davanzo, 2010), followed by a pilot clinical trial that demonstrated shorter treatment duration with clonidine than with morphine (Bada et al., 2015). Clonidine is an α_2 -adrenoceptor agonist commonly used as an antihypertensive agent (Nishina, Mikawa, Shiga, & Obara, 1999). In opioid withdrawal, enhanced noradrenergic activity is observed due to the loss of opiate inhibition on noradrenergic neurons (Bada et al., 2015). Through stimulation of α_2 -adrenergic receptors, clonidine decreases noradrenergic neurotransmission and thus relieves withdrawal symptoms (Bada et al., 2015; Langer, Caverro, & Massingham, 1980; Nishina et al., 1999).

A number of high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) methods that quantified morphine, M3G and M6G and those that quantified clonidine in human plasma were reviewed. The lowest limits of quantification (LLOQ) ranged from 0.25–3.5 ng/mL for morphine, 1–3.5 ng/mL for M3G/M6G (Clavijo et al., 2011; Eckart et al., 2015; Ghassabian et al., 2012), and 0.01–0.25 ng/mL for clonidine (Danafar & Hamidi, 2016; Muller et al., 2007; Nirogi, Kandikere, Mudigonda, & Komarneni, 2008; Parekh, Pudage, Joshi, Vaidya, & Gomes, 2008; Qin et al., 2015; Zhuang et al., 2015), and these methods required plasma volumes ranging from 100 μ L up to 1 mL. There was one published HPLC-MS/MS method that simultaneously quantified clonidine, morphine and its two major metabolites that was highly sensitive and only required 100 μ L of plasma (Veigure et al., 2017). However, this method required the use of an ion-pairing agent as the eluent additive, which could make column-to-column reproducibility problematic. Additionally, carry-over observed in this method, though sufficiently removed by extended needle and seal wash programs, was potentially problematic during method transfer to instruments from different manufacturers. On the other hand, compared to the protein precipitation method used in this particular study, solid-phase extraction (SPE) as the sample preparation method can yield cleaner sample extracts and more effectively reduce the amount of potential interferences from biological matrices.

Here, we report the development and validation of a HPLC-MS/MS method to simultaneously quantify morphine, M3G, M6G and clonidine in human plasma to support the pharmacokinetic analysis of an ongoing double-blinded randomized clinical trial, the Non-Opiate Treatment After Prenatal Opiate Exposure to Prevent Postnatal Injury to the Young Brain (No-POPPY) trial (NCT03396588), which compares the use of morphine versus clonidine in NAS. This assay is accurate, precise and only requires 100 μ L of patient plasma, considering only a limited amount of blood can be drawn from neonates. These data will be used for future population pharmacokinetic analyses, which aims to discover sources of variability in drug exposure and response to therapy including pharmacogenetic covariates identified in genes responsible for metabolism, drug transport and drug binding receptors.

2. Materials and methods

2.1 Chemicals and solvents

Reference standards of morphine solution (1.0 mg/mL in methanol), morphine-3- β -D-glucuronide solution (100 μ g/mL in methanol: water (1:1)), morphine-6- β -D-glucuronide solution (100 μ g/mL in methanol: water (1:1)), and clonidine hydrochloride were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). Deuterated internal standards (IS), clonidine- d_4 hydrochloride and morphine- d_3 solution (1.0 mg/mL in methanol) were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada) and Sigma-Aldrich, respectively. HPLC grade methanol (99.9%) and LC/MS grade formic acid (99.5%) were obtained from Fisher Scientific (Hampton, NH, USA). Ultrapure water was obtained from a Milli-Q water purification system (MilliporeSigma, Burlington, MA, USA). Pooled blank human plasma obtained from Kentucky Blood Center was used for method development and validation. Blank human obtained from six different healthy individuals with potassium EDTA as the anticoagulant was used to assess selectivity, LLOQ and matrix effect (Innovative Research, Novi, MI, USA).

2.2. Stock solutions, calibrators and quality control samples

Stock solutions of clonidine hydrochloride and clonidine- d_4 hydrochloride were prepared at 100 μ g/mL in methanol. All stock solutions were kept at -20°C until use. Working solutions for calibrators and quality control (QC) samples were made on the day of preparing the standards. Working solutions for calibrators were prepared by diluting stock solutions with ultrapure water to concentrations of 80, 40, 8, 4, 0.8, 0.4, 0.2 and 0.08 μ g/mL for morphine, M3G and M6G; 8, 4, 0.8, 0.4, 0.08, 0.04 and 0.02 μ g/mL for clonidine. Similarly, working solutions for QC samples at three concentrations (low [LQC], medium [MQC], high [HQC]) were prepared at concentrations of 0.24, 32 and 64 μ g/mL for morphine, M3G and M6G; 0.06, 3.2 and 6.4 μ g/mL for clonidine. IS working solution containing 2.5 μ g/mL of morphine- d_3 and 250 ng/mL of clonidine- d_4 hydrochloride was prepared freshly on the day of the experiment by diluting stock solutions with ultrapure water.

Calibrators and QC samples were prepared by spiking 95 μ L of blank human plasma with 5 μ L pooled working solutions from four analytes to achieve target concentrations. For morphine, M3G and M6G, calibrators 1–8 were prepared at 1000, 500, 100, 50, 10, 5, 2.5, 1

ng/mL, and QC samples were prepared at 3, 400, and 800 ng/mL. For clonidine, calibrators 1–7 were prepared at 100, 50, 10, 5, 1, 0.5, 0.25 ng/mL, and QC samples were prepared at 0.75, 40, and 80 ng/mL. A calibration curve for each analyte was tested using a zero sample, a blank sample, and nonzero calibrators.

2.3. Sample processing

Standards or 100 μ L of samples in polypropylene tubes were thawed under room temperature and centrifuged at 2000 g for 5 to 10 seconds. After centrifugation, 20 μ L freshly prepared IS working solution was added to each tube with the exception of zero samples, where 20 μ L of ultrapure water was added instead. Subsequently, 240 μ L of 10 mM ammonium carbonate (pH 10) was added to each tube. All samples were then vortexed for 5 seconds and centrifuged at 4000 g for 5 min at 4°C.

SPE was performed using Strata-X polymeric reversed phase 30 mg/mL tubes (Phenomenex, Torrance, CA, USA). Following loading of supernatant to SPE tubes conditioned with 1 mL methanol and 1 mL 10 mM ammonium carbonate (pH 10), the tubes were washed with 1 mL of methanol:10 mM ammonium carbonate (5:95 v/v) and dried under vacuum. Analytes were then eluted with 1 mL acetonitrile:methanol (1:1 v/v). The sample extracts were evaporated at 40 °C under vacuum and then reconstituted with 50 μ L 5% methanol in water with 0.1% formic acid (initial mobile phase). Reconstituted solutions were thoroughly vortexed for 10 seconds and centrifuged at 2000 g for 5 to 10 seconds. The supernatant was transferred to autosampler glass vials.

2.4. LC-MS/MS conditions

LC-MS/MS analyses were conducted with an HPLC system with SIL-20AC autosampler and LC-20AD pumps (Shimadzu, Tokyo, Japan), coupled with an API 2000 triple quadrupole mass spectrometer (AB Sciex, MA, USA). A C18 column (Inertsil ODS-3, 3 $\times$ 100 mm, 4- μ m, GL Sciences, Tokyo, Japan) coupled with a guard column (Inertsil ODS-3, 3 $\times$ 10 mm, 4- μ m) was used for analyte separation at room temperature. Mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in methanol (phase B). Flow rate was 0.2 mL/min, and the injection volume was 10 μ L. Temperature of the autosampler was kept at 15 °C. Mobile phase gradient started with 5% B, which was increased to 20% over 2 min and then to 40% over 0.5 min. Mobile phase B was held at 40% for 6 min, and then decreased to 5% over 0.5 min, which was held for 6 minutes for reconditioning. The total run time was 15 min, and the mass spectrum was recorded for the first 12 min of the run.

The mass spectrometer was equipped with a TurboIonSpray source, and the measurements were conducted in positive multiple reaction monitoring (MRM) mode. The following settings were used for the mass spectrometer: turbo heater temperature, 400 °C; curtain gas, 40 psi; gas 1, 35 psi; gas 2, 50 psi; collision activation dissociation (CAD) gas, 5 psi; ion spray voltage, 5500 V. The predominant ion transitions for each analyte and IS were selected for quantification, and the ion source parameters for each transition are listed in Table 1. Analyte peaks were quantified using Analyst software (version 1.4.2, AB Sciex). The analyte to IS peak area ratio was calculated and used for quantification of analyte

concentrations, with morphine-d₃ used as the IS for morphine, M3G and M6G, and clonidine-d₄ as the IS for clonidine.

2.5. Method validation

2.5.1. Linearity, lower limit of quantification (LLOQ) and limit of detection (LOD)—Linearity of analyte response was evaluated by analyzing two full sets of calibrators on three different days. Regression analysis of the calibration curves with $1/x^2$ weighting was performed in RStudio (version 1.1.442, RStudio). A standard on the calibration curve was accepted as the LLOQ if the analyte response at this concentration was at least five times the blank response, and the back-calculated concentration had accuracy within 20% of the nominal concentration and precision 20% when expressed as the coefficient of variation (CV%). The LOD was defined as the concentration at which the signal-to-noise ratio was at least 3. The calibration curve was accepted when 75% and a minimum of six non-zero standards did not deviate by >15% of nominal concentrations (or >20% at the LLOQ) (FDA). Carryover effect was assessed in blank plasma samples injected after the highest calibrators and the acceptable limit was below 20% of LLOQ.

2.5.2. Extraction recovery—The extraction recovery for each analyte was determined by comparing the peak area from processed QC samples in triplicates to the peak area from processed blank samples from six different sources that were spiked post extraction with diluted neat standards at low, medium and high concentrations. The absolute recovery of IS was evaluated in a similar manner at concentrations used in sample processing.

2.5.3. Selectivity and matrix Effect—Six different sources of blank plasma from healthy donors were used to test for interference, selectivity, and matrix effect. Selectivity was determined at the LLOQ, where the precision should be 20% and accuracy within 20% of the nominal concentrations. Matrix effect was evaluated by comparing the analyte peak area from processed blank samples from six different sources that were spiked post extraction with diluted neat standards at low, medium and high concentrations and analyte peak area from neat standards diluted in ultrapure water to the corresponding concentrations.

2.5.4. Intraday and interday precision and accuracy—Intraday and interday precision and accuracy were assessed by analyzing QC samples (LQC, MQC, HQC) in five replicates on three different days. Precision at each concentration was expressed as the CV %, and accuracy at each concentration was back calculated based on an averaged calibration curve from the method validation process. The performance of the assay was acceptable if intraday and interday precision was 15%, and the intraday and interday accuracy were within 15% of the nominal concentrations.

2.5.5. Stability—Plasma stability of the four analytes were evaluated by analyzing QC samples (N=3 at each concentration) under different storage and handling conditions. Freeze-thawing stability was tested by subjecting samples to three freeze-thaw cycles prior to processing. Bench-top stability was evaluated after 24 hours at room temperature, and short-term stability was determined after 6 days at 4°C. Long-term stability was tested after 1 month at -80 °C. Autosampler stability was assessed by analyzing processed QC samples

24 hours after the initial analysis. Stability of clonidine and clonidine-d₄ stock solutions was assessed after 24 hours at room temperature and after 1 month at -20 °C. Stability of morphine, M3G, M6G and morphine-d₃ stock solutions was determined based on certificates of analysis provided by the manufacturer. The testing samples were considered stable if back-calculated concentrations based on the averaged calibration curve were within 15% of the nominal concentrations.

2.6. Clinical samples

Blood samples for pharmacokinetic analysis were collected in EDTA tubes from subjects in the No-POPPY trial. Trial subjects were given oral doses of morphine or clonidine every 3 or 4 hours based on the feeding schedule. The starting dose was 0.06 mg/kg/dose for morphine and 1 µg/kg/dose for clonidine, and doses were adjusted based on subjects' Finnegan scores. Up to three blood samples were collected at random during treatment and two blood samples within 12 hours after discontinuation of therapy. Plasma was isolated by centrifugation at 2500 g at 15 min at 4 °C and stored at -80 °C until analysis. 30 plasma samples were analyzed by the aforementioned LC/MS-MS method in a single run to evaluate its applicability. Based on the US Food and Drug Administration (FDA) Draft Guidance for Bioanalytical Method Validation (FDA), three concentrations of QCs in duplicate were incorporated in the run, and the run was accepted if at least four out of six of the QCs concentration results were within 15% of their nominal values, and at least 50% of QCs at each concentration level were within 15% of their nominal values.

3. Results and Discussion

3.1. Method development

Solid phase extraction was adopted for effective clean-up and enrichment and its simplicity over liquid-liquid extraction, which was adopted by most of the published assays for clonidine (Nirogi et al., 2008; Qin et al., 2015; Zhuang et al., 2015). Both morphine and clonidine are both weakly basic with similar pK_a values (8.2 and 8.05, respectively) (Veigure et al., 2017). Therefore, two types of SPE processing protocols were compared, one using polymeric reversed phase sorbent (Strata-X, Phenomenex) with samples buffered with 10 mM ammonium carbonate at pH 10, and the other using strong cation mixed mode phase sorbent (Strata-X, Drug B, Phenomenex) with samples buffered with 10 mM ammonium acetate at pH 5. While comparable recovery of morphine was achieved by the two processing protocols, higher recovery of M3G/M6G was achieved with Strata-X. Therefore, Strata-X was selected as in the SPE method development.

Compound-dependent parameters on the mass spectrometer were manually optimized by directly injecting analytes and IS in 1 µg/mL methanol:water (1:1) solution, while ion source and gas settings were manually optimized by connecting the syringe pump and LC system to the source at a flow rate of 0.2 mL/min. For optimization of chromatography, as M3G and M6G have the same parent ion mass and the same MRM transition, LC condition was adjusted to achieve good separation of the two analyte peaks. Initial organic content of the mobile phase was kept low and gradient was kept shallow to improve the resolution between structurally similar morphine and its metabolites. Mobile phase gradient was further

adjusted to minimize matrix effect on the late-eluting clonidine and to optimize separation between endogenous interferences and the analytes. 0.1% formic acid was added into both the organic and aqueous mobile phases to reduce peak tailing. Satisfactory separation of the four analytes was achieved with the optimized LC condition. The expected retention times for morphine, M3G, M6G and clonidine were 5.5 min, 4.8 min, 6.5 min, and 7.3 min, respectively. The retention times for morphine-d₃ and clonidine-d₄ were 5.3 min and 7.3 min.

3.2. Method validation

3.2.1 Calibration curves—Calibration curves with eight concentrations prepared in duplicate on three different days showed good linearity, with R^2 from linear regression >0.99 in each run for all analytes. Average of calibration curves ($n = 6$) calculated by plotting the analyte/IS peak area ratio against the nominal concentration and applying $1/x^2$ weighted linear regression yielded the following equations: $y = 0.000131 + 0.001987x$, $y = 0.000111 + 0.008071x$, $y = 0.000353 + 0.005155x$, $y = 0.000559 + 0.01479x$ for morphine, M3G, M6G and clonidine, respectively. The mean accuracy calculated based on the averaged calibration curve and precision expressed in CV% were assessed at each calibrator level, and all standards met the predetermined acceptance criteria except for clonidine at 0.1 ng/mL. Therefore, the range of reliable response was 1–1000 ng/mL for morphine, M3G and M6G, and 0.25–100 ng/mL for clonidine. No carryover effect was observed for any of the analytes.

3.2.2. LLOQ and LOD—The lowest of calibrators were initially chosen based on expected plasma concentration ranges from the patients in the clinical study. After validation of the calibration curve, the lowest calibrators, i.e., 0.25 ng/mL for clonidine and 1 ng/mL for morphine, M3G and M6G were validated as the LLOQ in triplicates in six sources of plasma. The signal to noise ratio of all analytes at the LLOQ consistently exceeded 5 (Figure 1). For morphine, M3G, M6G and clonidine, the mean accuracy was 105.0%, 97.4%, 93.3% and 104.6%, and precision expressed in CV% was 14.3%, 10.2%, 14.1% and 14.9% at the LLOQ. CV% for analyte/IS area between each source of plasma ranged between 6.9% and 9.3%. Based on a S/N ratio of at least 3, the LOD was estimated to be 0.2 ng/mL, 0.05 ng/mL, 0.2 ng/mL and 0.15 ng/mL for morphine, M3G, M6G and clonidine, respectively.

3.2.3. Absolute recovery—Absolute recovery evaluated at three concentration levels are shown in Table 2. Absolute recovery was relatively consistent across concentrations tested, ranging from 84% to 94% for morphine, 92% to 104% for M3G, 84% to 95% for M6G, and 73% to 88% for clonidine. The absolute recovery for morphine-d₃ and clonidine-d₄ was 94% and 88%, respectively.

3.2.4. Selectivity and matrix effect—As shown in Table 2, the observed matrix effect was close to 100% at tested concentrations for morphine, M3G and M6G. There was potentially a small matrix effect on clonidine. However, as a stable isotope labeled internal standard was used for clonidine, matrix effect would have been compensated during analytical runs. The selectivity of the method was confirmed from the absence of interfering peaks in blank plasma (pooled plasma and six sources of single donor plasma) within the retention time window of the analytes and IS (Figure 2).

3.2.5. Intraday and interday precision and accuracy—Precision and accuracy data of the QC samples are presented in Table 3. The method showed intraday and interday CV% 15% for all analytes. Intraday and interday accuracy in the range of 95.4%–99.2% and 93.3%–97.1% for morphine, 90.4%–110.3% and 92.7%–111.3% for M3G, 97.3%–103.4% and 90.6%–100.8% for M6G, 102.5%–105.4% and 101.0%–108.9% for clonidine. The predetermined criteria of precision and accuracy were therefore achieved by this assay.

3.2.6. Stability—Based on the acceptance criteria of accuracy 15%, all analytes were stable in plasma after three freeze-thawing cycles. They were also stable in plasma for at least 24 hours at room temperature, 6 days at 4 °C, and for 1 month at –80 °C. Analytes in processed samples were stable in the autosampler for at least 24 hours (Table 4). Clonidine and clonidine-d₄ stock were shown to be stable for at least 24 hours at room temperature (91.3% and 106.9% of baseline) and 1 month at –20 °C (94.3% and 103.8% of baseline).

3.3. Application on clinical samples

Prior to each analysis, QC samples were analyzed to ensure that the system suitability criteria were met. Among the 30 plasma samples analyzed, 15 samples were collected from the morphine group and the remaining from the clonidine group. Among plasma samples collected from the clonidine group, 12 samples had concentrations ranging from 0.25 to 2.2 ng/mL (Figure 3A), while 3 samples (collected from the same subject) were below the LLOQ. The observed concentrations agreed with reported values in a previous pharmacokinetic study in newborns with NAS (Xie et al., 2011), and they corresponded to or exceeded concentrations that were found to achieve sedation in pediatric patients (0.3–0.8 ng/mL) (Sumiya et al., 2003).

Plasma samples collected from the morphine group during treatment (N=9) had morphine concentrations ranging from 1.3 to 32 ng/mL, M3G concentrations from 38 to 176 ng/mL, and M6G concentrations from 5.6 ng/mL to 36 ng/mL (Figure 3B). For the samples collected within 12 hours after drug discontinuation, morphine concentrations were mostly below 1 ng/mL, while M3G concentrations ranged from 1.1 to 16 ng/mL and M6G concentrations from <1 ng/mL to 4.2 ng/mL. The concentration range of morphine was similar to levels that were found to provide effective analgesia in postoperative neonates (between 15.4 and 22 ng/mL) (Bouwmeester, van den Anker, Hop, Anand, & Tibboel, 2003). Furthermore, the range of M3G/M6G concentration ratio (between 0.16 and 0.26) corresponded well to reported values in neonates (between 0.05 and 0.22) (Barrett, Barker, Rutter, Pawula, & Shaw, 1996; Bouwmeester et al., 2003). Representative chromatograms from study subjects are shown in Figure 4.

4. Conclusions

In this study, an accurate, precise and selective LC/MS-MS assay was developed and validated to quantify plasma concentrations of morphine, M3G, M6G and clonidine. This method was able to separate structurally similar glucuronide metabolites of morphine and minimize interference from endogenous compounds in plasma. Consistent and satisfactory recovery rates were achieved for all analytes, and the assay only requires a starting plasma volume of 100 µL. The method was successfully applied to clinical trial samples. The assay

has LLOQ of 1 ng/mL for morphine, M3G and M6G, and 0.25 ng/mL for clonidine. While the LLOQ of morphine, M3G and M6G were comparable to or lower than those in published methods, the LLOQ of clonidine was at the upper end of the reported range. Future work will be dedicated to lowering the LLOQ of clonidine by transferring the current method to a more sensitive mass spectrometry and performing necessary steps of partial validation for additional sensitivity needed for pediatric pharmacokinetic analysis.

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Figure 1.

Representative chromatograms of morphine at the LLOQ 1 ng/mL (top panel), morphine-3-glucuronide and morphine-6-glucuronide at the LLOQ 1 ng/mL (middle panel), and clonidine at the LLOQ 0.25 ng/mL (bottom panel) spiked in human EDTA plasma.

Figure 2.

Representative chromatogram of blank human EDTA single donor plasma in the MRM mode at five ion transitions (m/z 286.0 $\rightarrow$ 165.0, 289.4 $\rightarrow$ 165.0, 462.0 $\rightarrow$ 286.0, 230.1 $\rightarrow$ 44.3, and 234.2 $\rightarrow$ 48.3). Arrows represent expected retention times for analytes and internal standards (in the order of morphine-3-glucuronide, morphine/morphine- d_3 , morphine-6-glucuronide, clonidine/clonidine- d_4).

Figure 3. Concentrations of analytes measured in A) plasma samples above the lower limit of quantification from infants that received clonidine treatment and B) plasma samples collected during treatment from infants who were treated with morphine. The corresponding morphine metabolites are also depicted. A) Each point represents a single patient sample. B) Data points connected through a line represent concentrations from a single patient sample.

Figure 4.

Example chromatograms from patient samples in the morphine group (upper panel) and the clonidine group (lower panel). Patient sample numbers are hidden from the figure legend to maintain blinding of the clinical trial.

Table 1.

MRM transition and ion source parameters for analytes and internal standards.

Analyte	Parent mass (<i>m/z</i>)	Product mass (<i>m/z</i>)	DP (V)	FP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Morphine	286.0	165.0	30	395	10	14	56	10
Morphine-d ₃	289.4	165.0	30	395	10	14	56	10
M3G/M6G	462.0	286.0	63	395	10	22	45	10
Clonidine	230.1	44.3	33	395	10	14	47	27
Clonidine-d ₄	234.2	48.3	33	395	10	14	47	27

M3G: morphine-3-β-glucuronide; M6G: morphine-6-β-glucuronide; DP: declustering potential; FP: focusing potential; EP: entrance potential; CEP: collision cell entrance potential; CE: collision energy; CXP: collision cell exit potential.

Table 2-.

Absolute recovery and matrix effect for each analyte.

Analyte	Recovery (%)			Matrix Effect (%)		
	Low	Medium	High	Low	Medium	High
Morphine	99 ± 4	94 ± 7	96 ± 4	95 ± 16	90 ± 5	97 ± 5
M3G	90 ± 11	91 ± 8	98 ± 4	104 ± 6	101 ± 6	106 ± 3
M6G	98 ± 16	87 ± 9	101 ± 4	92 ± 13	97 ± 4	94 ± 4
Clonidine	79 ± 14	88 ± 6	93 ± 4	93 ± 5	85 ± 3	94 ± 9

M3G: morphine-3- β -glucuronide; M6G: morphine-6- β -glucuronide. Results are presented as mean $\pm$ standard deviation.

Table 3.

Precision and accuracy of QC samples.

	Analyte	Nominal Concentration (ng/mL)	Mean $\pm$ SD	Precision (%)	Accuracy (%)
Intraday (N=5)	Morphine	3	2.97 $\pm$ 0.39	14.9	99.2
		400	381.59 $\pm$ 8.52	2.3	95.4
		800	785.77 $\pm$ 16.69	2.1	98.2
	M3G	3	2.71 $\pm$ 0.15	5.9	90.4
		400	436.82 $\pm$ 32.69	7.5	109.2
		800	882.38 $\pm$ 22.92	2.6	110.3
	M6G	3	2.92 $\pm$ 0.24	10.2	97.3
		400	413.60 $\pm$ 35.26	8.5	103.4
		800	817.67 $\pm$ 33.24	4.1	102.2
	Clonidine	0.75	0.77 $\pm$ 0.07	12.8	102.7
		40	41.00 $\pm$ 0.99	2.5	102.5
		80	84.33 $\pm$ 3.13	3.8	105.4
Interday (N=15)	Morphine	3	2.80 $\pm$ 0.34	14.0	93.3
		400	373.67 $\pm$ 12.75	3.4	93.4
		800	776.99 $\pm$ 16.32	2.1	97.1
	M3G	3	2.78 $\pm$ 0.29	10.9	92.7
		400	423.89 $\pm$ 33.72	8.0	106.0
		800	890.32 $\pm$ 50.34	5.7	111.3
	M6G	3	2.72 $\pm$ 0.35	15.0	90.6
		400	403.31 $\pm$ 48.31	12.0	100.8
		800	789.73 $\pm$ 78.12	9.9	98.7
	Clonidine	0.75	0.82 $\pm$ 0.05	10.0	108.9
		40	40.41 $\pm$ 1.60	4.0	101.0
		80	81.96 $\pm$ 3.99	4.9	102.5

M3G: morphine-3- β -glucuronide; M6G: morphine-6- β -glucuronide.

Table 4.

Stability of analytes in various conditions.

Analyte	Nominal Concentration (ng/mL)	Accuracy (%)				
		24 h in autosampler	24 h at room temperature	6 days at 4 °C	1 month at –80 °C	Freeze-thawing
Morphine	3	110.9	113.7	115.0	93.0	91.5
	400	95.7	105.4	105.3	95.4	99.2
	800	100.3	103.4	110.1	96.4	102.6
M3G	3	100.0	98.3	93.0.1	104.1	103.5
	400	107.8	106.0	101.2	101.8	110.1
	800	112.2	113.0	113.0	99.7	110.5
M6G	3	101.4	112.8	109.8	95.7	102.1
	400	102.4	111.8	107.9	95.8	107.0
	800	101.5	106.1	111.0	87.6	97.1
Clonidine	0.75	95.8	98.5	97.8	101.7	110.2
	40	102.5	98.5	97.5	107.0	98.5
	80	105.4	96.8	99.2	111.5	102.2

M3G: morphine-3- β -glucuronide; M6G: morphine-6- β -glucuronide.