Additional file 1

SUPPLEMENTARY MATERIAL

Quantitative HPLC-MS/MS analysis of dexmedetomidine, oxycodone, noroxycodone, oxymorphone and noroxymorphone in human plasma

Chemicals and supplies. Dexdor® dexmedetomidine infusion concentrate was from Orion (Espoo, Finland) and the internal standard medetomidine-d3 hydrochloride was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Oxycodone, noroxycodone, oxymorphone, noroxymorphone, oxycodone-D3, noroxycodone-D3 and oxymorphone-D3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS-grade methanol was purchased from Honeywell / Riedel-de Haën (Seelze, Germany), and LC-MS grade formic acid was purchased from VWR International ((Fontenay-Sous-Bois, Paris, France). Ammonium acetate and 25 % ammonia solution were purchased from Merck (Darmstadt, Germany). Water used was purified with Millipore Synergy UV (Merck KGaA, Darmstadt, Germany) system.

Preparation of calibration standards and quality control samples. The dexmedetomidine concentrate (100 μgmL^{-1}) served as stock solution for dexmedetomidine. Stock solution of the internal standard medetomidine-d3 hydrochloride (100 µg/ml) was prepared in methanol/H_2O (1:1, v/v). The stock solutions were further diluted with H_2O to obtain required working solutions. For preparation of calibration standards at 9 concentration levels and quality control samples at 4 concentration levels, appropriate volumes of working solutions were mixed with pooled human K_2EDTA plasma. The dexmedetomidine concentrations of the calibration standards were 0.02, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 4.5 and 5.0 ng/mL, and the concentrations of the quality control samples were 0.06, 0.15, 1.0 and 4.0 ng/mL. Standards, controls and actual study samples were stored at -70 °C and processed in an identical manner.

Oxycodone, noroxycodone, oxymorphone and noroxymorphone stock solutions and working solutions were prepared in methanol. Working solutions contained either all four analytes or all three internal standards. Calibration standards and quality control (QC) samples were prepared by spiking pooled, drug-free human K_2EDTA plasma with appropriate volumes of working solutions. The calibration standards contained oxycodone, noroxycodone, oxymorphone, and noroxymorphone at 0.05, 0.1, 0.3, 2.0, 10.0, 20.0, 45.0, and 50.0 ng/mL. QC concentrations were 0.15, 12.0, and 40.0 ng/mL. For oxycodone, additional dilution controls of 200 ng/mL (10× dilution) and 2000 ng/mL (100× dilution) were prepared for selected batches. All calibration standards, QC samples, and study samples were stored at –70 °C until analysis and processed identically.

Sample preparation. For the analysis of dexmedetomidine, sample preparation was performed using solid phase extraction. Aliquots of 250 µl of plasma were mixed with 675 µl of 0.1 % formic acid in water (v/v) and 75 µl of internal standard solution (medetomidine-d3, 1 $ngmL^{-1}$). Samples were then extracted with Sep-Pak® tC18 100 mg 96-Well Plates (Waters Corporation, Milford, MA, USA) using an Oasis 96-well plate extraction manifold (Waters). The evaporation residue was dissolved in 100 µl of methanol/H_2O (7/3, v/v). Final samples were transferred into 200 µl autosampler vials, and the vials were centrifuged.

For oxycodone, noroxycodone, oxymorphone and noroxymorphone SPE was also used for sample preparation. Briefly, 200 µL of plasma was mixed with 25 µL of internal standard working solution (100 ng/mL) and 775 µL of 0.1 % formic acid in water (v/v). After vortex-mixing, samples were transferred to conditioned Sep-Pak® tC18 96-well plates (Waters, Milford, MA, USA). Analytes were eluted with 1 mL methanol, evaporated under nitrogen, and reconstituted in 100 µL of 20 % methanol in water (v/v). The extracts were transferred to autosampler vials, centrifuged at 7500 × g for 2 min, and subjected to HPLC–MS/MS analysis.

HPLC-MS/MS analysis and quantitation. The HPLC-MS/MS system used in sample analysis consisted of a Sciex ExionLC™ AD HPLC instrument and a Sciex QTrap 6500^{+} triple quadrupole mass spectrometer (AB Sciex LP, Concord, ON, Canada). For the analysis of dexmedetomidine samples, chromatographic separations were performed with a Gemini C18 analytical column (150 x 2.0 mm, particle size 5 µm) coupled with a Gemini C18 precolumn from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 0.1 % HCOOH in water (A) and 0.1 % HCOOH in methanol (B) with a constant flow rate of 0.3 mL min^{-1} with the following gradient: 0–1 min: 10 % B; 1–7 min: 10–95 % B (linear gradient); 7.0–7.5 min: 95–10 % B (linear gradient); 7.5–11 min: 10 % B. The injection volume was 15 µL. The column oven temperature was + 28 °C. Mass spectrometric detection was carried out using positive Turbo Ion Spray (TIS) ionization and multiple reaction monitoring (MRM) mode. The MRM transition for dexmedetomidine was 201.2 > 95.05 and for medetomidine-d3 204.2 > 98.05. The declustering potential was 55 V for both molecules, entrance potential was 9 V, collision energy was 25 V and collision cell exit potential was 7. The ion source temperature was + 550 ºC, and the nebulizer gas (Gas 1) and turbo gas (Gas 2) settings were set at 50. Curtain gas (nitrogen) was set at 25, TIS voltage was 5100 V and dwell time was 250 ms.

For oxycodone, noroxycodone, oxymorphone and noroxymorphone, chromatographic separation was performed with a Kinetex™ EVO C18 analytical column (50 x 4.6 mm, particle size 5 µm) coupled with an EVO C18 guard column from Phenomenex (Torrance, CA, USA). The mobile phase consisted of (A) 5 mM ammonium acetate in water, adjusted to pH 9.4, and (B) 100 % methanol, delivered at a constant flow rate of 0.45 mL/min. The gradient was as follows: 0.0–1.0 min, 10 % B; 1.0–2.0 min, linear increase to 40 % B; 2.0–5.0 min, 40 % B; 5.0–6.0 min, linear increase to 95 % B; 6.0–8.0 min, 95 % B; 8.0–8.1 min, linear decrease to 10 % B; and 8.1–10.0 min, 10 % B. The injection volume was 10 µL. The column oven temperature was + 50 °C. Mass spectrometric detection was carried out using positive Turbo Ion Spray (TIS) ionization and single reaction monitoring (SRM) mode. The SRM-transitions and ion specific parameters are summarized in Table 1. The ion source temperature was 500 °C. The nebulizer gas (Gas 1) and turbo gas (Gas 2) settings were 50 psi. Curtain gas (nitrogen) was set to 20 psi. The TIS voltage setting was 4500 V. Dwell time was 250 ms for all ion transitions.

Table 1. SRM transitions and ion-specific parameters used for HPLC–MS/MS quantitation.

Analyte	**Transition**	**Declustering Potential (V)**	**Entrance Potential (V)**	**Collison Energy (eV)**	**Collision Cell Exit Potential (V)**
oxycodone	316 > 241	60	10	39	7
noroxycodone	302 > 227	60	10	39	10
oxymorphone	302 > 227	60	10	39	10
noroxymorphone	288 > 213	70	12	39	12
oxycodone-D_3	319 > 244	60	10	39	7
noroxycodone-D_3	305 > 230	60	10	39	10

oxymorphone-D_3	305 > 230	60	10	39	10

Quantitation was based on the peak area ratios of the analyte to the internal standard. Data from the HPLC-MS/MS analyses were acquired using AB Sciex Analyst 1.6.3 software. Peak integration, calibration curves, and quantitation were generated with the same software. Statistical analyses were performed with Microsoft Excel 2016 software. The calibration curves were fitted using linear regression with a $1/x^2$ weighting factor.

Acceptance criteria. For the study sample analysis, the back-calculated concentrations of the calibration standards had to be within ± 15 % of the nominal values (± 20 % at LLOQ) and at least 6 of 8 calibration standards were to fulfill the criterion. The accuracy of the QC samples had to be ± 15 % of the nominal values and at least 50 % at each concentration level and 4/6 from all QC samples had to fulfill the criterion. The concentration results for dexmedetomidine were acquired in ten analysis batches. The inter-assay accuracies of the QC samples were (n= 18) 97.1 % – 105 % and the corresponding inter-assay coefficients of variation were 2.42 % – 7.54 %. The concentration results for oxycodone, noroxycodone, oxymorphone and noroxymorphone were acquired in ten analysis batches. The inter-assay accuracies of the QC samples were (n= 20 – 28) 89.8 % – 109 % for oxycodone, 87.8 % – 105 % for noroxycodone, 89.1 % – 105 % for oxymorphone and 93.2 % – 103 % for noroxymorphone. The corresponding inter-assay coefficients of variation were 4.44 % – 13.5 %, 5.25 % – 10.2 %, 3.27 % – 10.1 %, and 4.54 % – 11.6 %, respectively.