

Simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and ketodoxapram in plasma using liquid chromatography – tandem mass spectrometry

Robert B. Flint^{1,2,3}, Soma Bahmany¹, Bart C.H. van der Nagel¹, Birgit C.P. Koch¹

1. Department of Pharmacy, Erasmus University Medical Center, Rotterdam, the Netherlands
2. Department of Pediatrics, Division of Neonatology, Erasmus University Medical Center - Sophia, Rotterdam, the Netherlands
3. Department of Pharmacy and Radboud Institute of Health Sciences (RIHS), Radboudumc, Nijmegen, The Netherlands

Corresponding author

R.B. Flint

Erasmus University Medical Center

Department of Pharmacy

Postal Box 2040

3000 CA Rotterdam

The Netherlands

Email: r.flint@erasmusmc.nl

Phone: +31 107033202

Fax: +31 107032400

Funding information

This study was enabled by funding from the Netherlands Organisation for Health Research and Development ZonMw (Grant number: 80-83600-98-10190).

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/bmc.4290

ABSTRACT

A simple and specific UPLC-MS/MS method was developed and validated for simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and its active metabolite keto-doxapram. The internal standard was fentanyl-d5 for all analytes. Chromatographic separation was achieved with a reversed phase Acquity UPLC HSS T3 column with a run-time of only 5.0 minutes per injected sample. Gradient elution was performed with a mobile phase consisting of ammonium acetate, formic acid in Milli-Q ultrapure water or in methanol with a total flow rate of 0.4 mL minute⁻¹. A plasma volume of only 50 µL was required to achieve both adequate accuracy and precision. Calibration curves of all 5 analytes were linear. All analytes were stable for at least 48 hours in the autosampler. The method was validated according to US Food and Drug Administration guidelines. This method allows quantification of fentanyl, sufentanil, cefazolin, doxapram and keto-doxapram, which serves purposes for research, as well as therapeutic drug monitoring, if applicable. The strength of this method is the combination of a small sample volume, a short run-time, a deuterated internal standard, an easy sample preparation method and the ability to simultaneously quantify all analytes in one run.

Key words

UPLC-MS/MS, fentanyl, sufentanil, cefazolin, doxapram

1. INTRODUCTION

One of the most important issues in peri-operative and intensive care medicine is the establishment of an individual antibiotic and analgesedation drug profile for each patient with respect to the clinical situation, together with support of vital functions. In general, the analgesic and sedation dose regime will be adjusted to the clinical situation of each individual patient to shorten duration of therapy and to reduce morbidity (Chanques et al., 2006). Large knowledge gaps still exist with respect to the optimal drug therapy and covariates that determine the effect and safety, especially in neonates and infants, which requires well designed trials (Coppini, Simons, Mugelli & Allegaert, 2016).

For certain drugs therapeutic drug monitoring (TDM) has been proven valuable to ensure drug effects, dosage regimes, monitor physiological changes, and when appropriate, adapt the medical health care of each patient in the intensive care unit. Despite many drugs are still being dosed on clinical response, the continuously expanding assortment of analytical methods improves drug safety and individual patient treatment (Touw, Neef, Thomson & Vinks, 2005). Both for research as well as for TDM, more assays are necessary with a minimal sample volume, a short runtime, quick and easy sample preparation, and simultaneously measuring multiple analytes. Simultaneous quantification in one assay either allows to quantify multiple analytes in one sample without requiring extra sample volume, and allows to run samples containing different drugs efficiently in one assay-run.

Evidence is sparse on the use of fentanyl, sufentanil, cefazolin and doxapram for certain pediatric age-ranges and indications. Sufentanil, fentanyl and cefazolin are part of peri-operative treatments for children. Furthermore, sufentanil and fentanyl are synthetic opioid analgesics widely used in clinical anesthesia and analgesia (Mather, 1983; Pacifici, 2015). Cefazolin is a first-generation cephalosporin Beta-lactam antibiotic used for treatment of sepsis or life-threatening infections (McWhinney et al., 2010), where adequate individual dosing may be lifesaving. Neither has doxapram been investigated sufficiently in children, despite its frequent and promising use in neonatal intensive care for treatment of apnea of prematurity (Flint et al., 2017a; Flint et al., 2017b; Pacifici, 2015; Prins, Pans, van Weissenbruch, Walther & Simons, 2013).

This assay will serve future research to close the knowledge gaps on these four drugs, but may also be used for therapeutic drug monitoring (TDM) if a target concentration range may be defined. As these four drugs are commonly prescribed and combined, we aimed to develop

and validate a quick and easy analytical method for simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and its active metabolite keto-doxapram in human plasma by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-MS/MS). We optimized sensitivity of the assay so to minimize the required sample volume, which allows measurement of small volume samples, even from premature born infants.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Fentanyl, fentanyl-d5 and sufentanil were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Cefazolin was obtained from Santa Cruz Biotechnology (Heidelberg, Germany), doxapram from Selleckchem (Munich, Germany) and keto-doxapram from Tractus (London, England). Ammonium acetate was obtained from Sigma Aldrich (Zwijndrecht, the Netherlands). Methanol, acetonitrile and formic acid were purchased from Biosolve BV (Valkenswaard, the Netherlands). All reagents were LC-MS grade, which means at least 99% purity. Water was purified by using a MilliPore Advantage A10 system. Human drug-free plasma was obtained from the blood donation center (Sanquin, Rotterdam, the Netherlands).

2.2 Stock solutions, calibration standards, quality control samples and internal standard

Stock solutions of doxapram and keto-doxapram were prepared at a concentration of 500 mg L⁻¹ using methanol. The following substance stock concentrations in methanol were prepared, sufentanil at a concentration of 20 mg L⁻¹, fentanyl at a concentration of 2 mg L⁻¹, and cefazolin at a concentration of 5000 mg L⁻¹ using Milli-Q water. For each analyte two separate stock solutions were made with the same concentration, for both calibration of standard samples and for quality control samples. Stock solutions were stored at -20°C, except the stock solution of cefazolin which was stored at 2-8°C. The calibration standard 8 and Quality Control (QC) High were made from the stock solutions with drug-free human plasma. Calibration standard 1 through 7 and the lower limit of quantification (LLOQ) standard were prepared by serial diluting of calibration standard 8 with human plasma. QC Medium and QC High samples were prepared the same way, using the other stock solution (QC High) which was diluted with human plasma. The concentrations of all calibration standards are given in Table 1 and the concentrations of the quality controls are given in Table 2. Calibration standards and quality

control samples were stored as 50 μL portions in 1.5 mL Eppendorf tubes at -80°C prior to analysis. The internal standard was fentanyl-d5, which was dissolved in a mixture of acetonitrile and methanol 1:1 at a concentration of $10\ \mu\text{g L}^{-1}$. The internal standard working solution was stored at -20°C .

2.3 Sample preparation

A mixture of acetonitrile and methanol, containing $10\ \mu\text{g L}^{-1}$ fentanyl-d5 (the internal standard solution) was used for protein precipitation. 50 μL of the calibration standards, quality control samples, blanks and patient samples were thawed at least half an hour prior to preparation. Then plasma proteins were precipitated by adding 200 μL of the internal standard solution. Subsequently, the samples were vortexed for about 10 seconds. After vortexing, the precipitant was separated by centrifugation for 5 minutes at $16,000 \times g$. 100 μL of each supernatant was transferred into an auto sampler insert vial (VWR, Amsterdam, the Netherlands) and diluted by adding 400 μL of mobile phase A. The autosampler vials were mixed by using the vortex for 10 seconds. For cefazolin, doxapram and keto-doxapram 1 μL was injected into the UPLC. For fentanyl and sufentanil, 10 μL was injected into the system because of the lower therapeutic range of these compounds (see Table 2).

2.4 Instrumentation

A Dionex Ultimate UPLC system consisting of an Ultimate 3000 RS UPLC pump, an Ultimate 3000 RS autosampler and an Ultimate 3000 RS Column Compartment were used as the equipment. The UPLC was connected to a Thermo TSQ Vantage triple quadrupole MS with HESI probe (Thermo Scientific, Waltman, MA). The software programs Chromeleon (version 6.8, Dionex, Thermo Scientific), Xcalibur (version 2.1, Thermo Scientific), and LCQuan (version 2.6, Thermo Scientific) were used to control the system and analyze the data.

2.5 UPLC conditions

Chromatographic separation, based on affinity of the analytes with the nonpolar stationary phase, was achieved with a reversed phase UPLC Acquity BEH C18 column, $1.7\ \mu\text{m}$, $2.1 \times 100\ \text{mm}$ (Waters, Milford, USA). Gradient elution was performed with a mobile phase consisting of 1 mL of a 154 mg/L solution of ammonium acetate in formic acid (99%) in 1 L of Milli-Q ultrapure water (eluent A) and 1 mL of the same solution in 1 L of methanol (eluent B). Before the analysis, the system was equilibrated at the starting conditions of 75% eluent A and 25% eluent B until pressure was stable. The multistep gradient was as follows: from 0 to 0.6 minutes,

eluent B was increased from 25% to 48%; from 0.6 to 1.5 minutes, eluent A decreased to 35% and B was increased to 65%; from 1.6 to 2.8 minutes, eluent B was kept stable at 100% and 0% eluent A; from 3.0 to 5.0 minutes, eluent A was increased to 75% and B was decreased to 25%. The run ended at 5.0 minutes at starting conditions. Temperature for the column oven was set at 50 °C and for the autosampler at 15 °C.

The separation was performed by gradient elution using mobile phase A (1 mL of 2M ammonium acetate in formic acid 99%), in 1 L Milli-Q water and mobile phase B (1 mL of 2M ammonium acetate in formic acid 99%), in 1 L methanol with a total flow rate of 0.4 mL min⁻¹. Mobile phase B was kept at 25% from 0.0 min to 0.6 min, then at 48%, from 0.6 to 1.5 min mobile phase B at 65%, then from 1.6 to 2.8 min at 100%, from 3.0 to 5.0 min mobile phase B was kept at 25%. The run ended at 5.0 minutes at starting conditions. Temperature for the column oven was set at 50 °C and for the autosampler at 15 °C.

2.6 MS/MS conditions

MS/MS detection was performed in positive mode by using Selected Reaction Monitoring (SRM) with electrospray ionization. To optimize the MS/MS parameters to detect the most intense signal of each analyte, solutions of 1 mg L⁻¹ were directly infused in methanol by addition of the mobile phase (75% mobile phase A and 25% mobile phase B) from the LC at a flow rate of 0.4 mL min⁻¹. The MS/MS instrument was operated with a capillary spray voltage of 3 kV, vaporizer temperature at 375 °C, capillary temperature at 250 °C, sheath gas pressure at 50 (arbitrary units), auxiliary nitrogen gas pressure at 20 (arbitrary units), collision gas pressure at 1.5 mTorr. Specific parameters for each compound are given in table 3.

2.7 Assay Validation

Validation of the method was performed according to the US Food and Drug Administration (2001) guidelines for bio analytical methods (FDA, 2003). The following validation parameters were investigated; linearity, LLOQ and ULOQ, accuracy, repeatability, reproducibility, stability and matrix effect.

Linearity

To investigate the linearity of the method, a blank sample (without internal standard), a zero sample (blank with internal standard) and eight calibration standards in duplicate were prepared and analyzed. Calibration curves were generated by plotting the theoretical standard

concentration versus the ratio of the standard peak area to the internal standard area. The determination coefficient (R^2) should be at least 0.9950. The relative standard deviation (RSD) of the calculated concentrations of the standard concentrations was required to be lower than 15%, except at the LLOQ, where it should not deviate by more than 20%. It was decided to apply weighting $1/x$, which means that standards with the lowest concentrations are more important for the calibration line than standards with highest concentrations (Saar, Gerostamoulos, Drummer & Beyer, 2010). The calibration curves were formed using the peak area ratios for the analytes and their corresponding internal standard (response) versus the concentrations applying linear least square regression with a weighing factor of $1/x$ and excluding of the origin.

LLOQ and ULOQ

The LLOQ was measured by analyzing the LLOQ standard six times in a row. Mean and standard deviation of the response ratios of the six samples were measured. The response of the analyte should be at least 5 times the response compared with the response of the blank. Precision and accuracy were calculated and should be $\leq 20\%$ and the accuracy should be between 80-120%, respectively. The highest standard of the calibration curve was used as upper limit of quantification (ULOQ).

Accuracy

Accuracy was measured by measuring three concentrations (QC-H, QC-M and QC-L) in six-fold on the same day. The difference in percentage, between the measured concentration and the theoretical concentration, known as the relative standard deviation (RSD) was required to be lower than 15%.

Repeatability and reproducibility

The repeatability was tested by analyzing three QC levels in six-fold on the same day. The reproducibility was tested by analyzing three concentrations in duplicate on six different days. The requirement for both parameters was an RSD lower than 15%.

Stability

Autosampler stability was determined by storing QC samples ($n=2$ per concentration) after sample preparation in the autosampler for 24, 48, 72 and 120 hours. Response ratios were measured and compared with response ratios of samples kept at -80°C prior to preparation.

After sample preparation, samples were directly analyzed. Recovery was required to be between 90 and 110%.

Matrix effect

It is important to measure matrix effects and absolute recoveries in the development of an LC-MS/MS method since ion suppression and ion enhancement effects can be expected owing to interferences by matrix compounds, stable-isotope-labeled internal standards and co-eluting compounds (Van Eeckhaut, Lanckmans, Sarre, Smolders & Michotte, 2009). In order to check whether the precision, the reproducibility and the stability of the concentration-signal ratio are affected by interference of the matrix analytes, the method described by Matuszewski et al. was used (Matuszewski, Constanzer & Chavez-Eng, 2003). Five different lots of human plasma were used. To two concentration levels (QC Low and QC High) (both in duplicate), the analytes were added before and after extraction, which served to calculate the recovery. Also, a set of six academic samples was evaluated with only Milli-Q ultrapure water instead of plasma. Matrix effects were calculated as follows:

$$\frac{\text{peak area of analyte spiked after extraction}}{\text{peak area of analyte prepared in Milli-Q ultrapure water}} \times 100\%$$

The process efficiency was calculated as the percentage ratio of the area of the analytes spiked before extraction and the ones prepared in Milli-Q ultrapure water. The mean and RSD were calculated of both matrix effects, process efficiency and recovery. In the ideal situation, the mean matrix effects, process efficiency and recovery are between 80% and 120%, and the RSD of both parameters is $\leq 15\%$. Furthermore, for each analyte, the internal standard normalized matrix effect should also be calculated by dividing the matrix effect of the analyte by the matrix effect of the IS. The RSD of the internal standard-normalized matrix effect calculated from the different lots of matrix should not be greater than 15%.

2.8 Clinical application

The method was developed for the analysis of plasma samples from a pharmacokinetic study and may also be used for TDM, if this can be proven clinically valuable. For the validation of the assay for clinical practice, clinical application, and research purposes the method has been applied to quantify doxapram, keto-doxapram and fentanyl in plasma of preterm born infants participating in a clinical study. The Erasmus Medical Center ethics review board approved the protocol and written informed consent from parents/legal guardians was obtained prior to study initiation (MEC-2014-067, ClinicalTrials.gov by NCT02421068). This observational

prospective multicenter study was performed between September 2014 and June 2017 at the Departments of Neonatology of the Radboud University Medical Centre in Nijmegen, Maastricht University Medical Centre in Maastricht, Maxima Medical Centre in Veldhoven and Sophia Children's Hospital in Rotterdam. Neonates routinely received doxapram (Dopram®, Manage, Belgium) for treatment of apnea of prematurity starting with a loading dose of 2.5 mg kg⁻¹ bodyweight in 15 minutes, followed by a maintenance starting dose of 2.0 mg kg⁻¹ h⁻¹, either by continuous intravenous infusion or continuous gastro-enteral administration. Fentanyl (Bipharma, Almere, the Netherlands) was indicated for comfort as an intravenous continuous infusion of 0.5 – 2.0 µg kg⁻¹ h⁻¹ or as a bolus injection of 0.5 – 3.0 µg kg⁻¹.

3. RESULTS

3.1 Linearity

Linearity was achieved for each analyte in the range between the LLOQ and the ULOQ (Table 2), with all RSD's to be lower than 15% and the determination coefficient (r^2) to be at least 0.995. The calibration curves showed that a regression with a weighting factor of 1/x best described the data set over the range for all analytes. Figure 1 shows the ion chromatograms obtained after the analysis of the lowest plasma calibrator standard for all the analytes, and the corresponding retention times of each analyte (see Table 1).

3.2 LLOQ and ULOQ

The results for the LLOQ for cefazolin, keto-doxapram and fentanyl did not meet the initial requirements. Therefore, the LLOQ for these analytes was set to calibration standard 1, which was acceptable. The results of determination of LLOQ and ULOQ are shown in Table 4.

3.3 Accuracy, repeatability and reproducibility

The RSD of accuracy, repeatability and reproducibility data were within the requirement of an RSD lower than 15% (Table 4).

3.4 Stability

Except for cefazolin and keto-doxapram, the recovery of all QC's was between 90% and 110%, indicating that they were stable for at least 120 hours when stored in the autosampler at 15 °C. Cefazolin was only stable for 72 hours and keto-doxapram only for 48 hours.

3.5 Matrix effect

Matrix effects and absolute recoveries in the development of the LC-MS/MS method are shown in Table 5. The method described by Matuszewski et al. showed that fentanyl, sufentanil, cefazolin, doxapram and keto-doxapram experienced neither matrix effect, nor an effect from the sample preparation. A good recovery was achieved for all analytes.

3.6 Clinical application

A total of 618 samples were collected from a pediatric cohort of preterm born infants (n = 157), consisting of 92 infants who received fentanyl and 65 infants with doxapram. Eleven samples were collected from six patients from the cohort of 157 infants who received fentanyl and doxapram simultaneously. The median gestational age of the fentanyl cohort was 27.1 weeks (range 24.3 - 31.2 weeks), median postnatal age at start of drug therapy 4.5 days (range 0 - 68 days), median body weight at start of drug therapy 968 g (range 465 - 3000 g). The median gestational age of the doxapram cohort was 26.1 weeks (range 24.0 - 29.4 weeks), median postnatal age at start of drug therapy 17 days (range 1 - 52 days), median body weight at start of drug therapy 960 g (range 650 - 1520 g).

Fentanyl was quantified in 370 samples from 92 patients, and doxapram and keto-doxapram in 248 samples from 65 patients. For fentanyl 78 (21%) of the 370 samples was measured below LLOQ, and 19 (5%) below LOD. For doxapram 29 (12%) and for keto-doxapram 33 (13%) of the 248 samples were below LLOQ, and 8 (3%) doxapram and 6 (2%) keto-doxapram measurements were below LOD. For doxapram, three (1.2%) samples were measured above the ULOQ.

4. DISCUSSION

We developed a robust UPLC-MS method for simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and its active metabolite keto-doxapram according to US Food and Drug Administration guidelines. The easy sample preparation, small required sample volume of 50 μ L human plasma and short runtime of 5.0 minutes perfectly met the objectives. Herewith, we are able to analyze one plasma sample to simultaneously quantify multiple drugs that are part of one treatment, and combine samples with different drugs to be measured in one assay run.

Previously reported methods for quantification of these analytes concerned either one of these analytes (with or without their metabolites) per assay, or in a combination with other drugs.

These combinations mostly concerned multiple drugs from the same drug class; i.e. sufentanil or fentanyl with other analgesedatives by Nosseir et al. (Nosseir, Michels, Binder, Wiesen & Muller, 2014) and by Fernandez et al. (Fernandez Mdel et al., 2013), cefazolin with beta-lactams by Carlier et al. (Carlier et al., 2012) and Kiriazopoulos et al. (Kiriazopoulos et al., 2017). Our assay concerns four drugs from three different Anatomical Therapeutic Chemical (ATC) classes; fentanyl and sufentanil as nervous system drugs, cefazolin as an anti-infective drug, and doxapram as a respiratory drug. Herewith, our assay enables to quantify four drugs in one sample simultaneously following one sample injection. This may be valuable for TDM as well as for research, concerning patients using a combination of these drugs as part of a certain treatment protocol. The burden to the patient may be reduced compared to a separate assay per drug, which is especially important concerning vulnerable (preterm) infants. Furthermore, samples with different drugs may be combined in one single run, which may improve efficiency of the laboratory process.

In general, for all analytes, our assay performed better than or comparable to prior reported assays, even in comparison with assays measuring only a single analyte, which makes it easier to achieve good performance on runtime, required sample volume, matrix effects (Aranda, 1988; Carlier et al., 2012; Clavijo et al., 2011; Fernandez Mdel et al., 2013; Hisada et al., 2013; Kiriazopoulos et al., 2017; LeGatt, Beaudry & Bradley, 1986; Lilloco et al., 2016; Mahlke, Ziesenitz, Mikus & Skopp, 2014; Nichol, Vine, Thomas & Moore, 1980; Nosseir, Michels, Binder, Wiesen & Muller, 2014; Palleschi et al., 2003; Parker et al., 2016; Robson & Prescott, 1977; Saari, Fechner, Ihmsen, Schuttler & Jeleazcov, 2012; Suzuki et al., 2017). Furthermore, most assays use a different drug as an internal standard, where we used a deuterated form of fentanyl, which shows better comparable behavior to the analytes that are measured than using a different drug. Next, our sample preparation consisted of a simple one-step protein precipitation method, where in most studies a solid phase extraction is prescribed, or a liquid-liquid extraction with an evaporation and/or ultrafiltration step, or other additional steps.

For sufentanil, other reported assays required more plasma volume and a more complex sample preparation compared to our assay (Nosseir, Michels, Binder, Wiesen & Muller, 2014; Palleschi et al., 2003; Saari, Fechner, Ihmsen, Schuttler & Jeleazcov, 2012).

Concerning doxapram, four of the five reported assays date from the 90's (Aranda, 1988; LeGatt, Beaudry & Bradley, 1986; Nichol, Vine, Thomas & Moore, 1980; Robson & Prescott, 1977) and are inferior to our assay with respect to the use a different drug for

internal standard, sample preparation which requires an evaporation step, higher LLOQ, larger sample volume required, a longer runtime, and two assays could not measure keto-doxapram. The recently published assay by Suzuki et al. (Suzuki et al., 2017) required only 25 μL plasma volume compared to our 50 μL , and an LLOQ for doxapram of 20 $\mu\text{g L}^{-1}$ compared to our 50 $\mu\text{g L}^{-1}$. On the other hand, Suzuki et al. needed a 12 minutes runtime and used propranolol an internal standard, where we needed 5 minutes runtime and used deuterated fentanyl, and our sample preparation required less operational steps. Regarding cefazolin, multiple assays have been reported with comparable performance (Carlier et al., 2012; Kiriazopoulos et al., 2017; Lillico et al., 2016; Parker et al., 2016). The reported fentanyl assays require larger sample volumes except for Hisada et al., who need only 20 μL and reached a lower LLOQ of 0.05 $\mu\text{g L}^{-1}$ compared to our 0.1 $\mu\text{g L}^{-1}$ (Hisada et al., 2013). Except for cefazolin and keto-doxapram, the stability of all analytes was good, which means they were stable for at least 120 hours when stored in the autosampler at 15°C. Cefazolin was only stable for 72 hours and keto-doxapram only for 48 hours at 15°C. Suzuki et al. tested the stability of keto-doxapram for 48 hours at 10°C for autosampler conditions and also found them to be stable for that time (Suzuki et al., 2017). Stability during 3 cycles of freezing and thawing, together with freezer stability studies of the analytes in the matrix are not presented because they were already carried out in previous published papers and do not depend on the analytical method. No relevant effects of freezing and thawing were found for all analytes, together with good stability at -20°C for 4 weeks (Clavijo et al., 2011; Kiriazopoulos et al., 2017; Mahlke, Ziesenitz, Mikus & Skopp, 2014; Nosseir, Michels, Binder, Wiesen & Muller, 2014; Palleschi et al., 2003; Parker et al., 2016; Saari, Fechner, Ihmsen, Schuttler & Jeleazcov, 2012; Suzuki et al., 2017).

Our assay fulfilled the desired criteria for accuracy, repeatability, and reproducibility. Furthermore, for all analytes a good recovery was achieved and matrix effects were measured. These indicated the absence of interferences by matrix compounds, stable isotope-labeled internal standard, and co-eluting compounds, that may cause ion suppression and ion enhancement.

The ranges for linearity for all analytes were perfectly suitable for clinical pharmacology research, as well as for possible TDM purposes. The assay was successfully validated for clinical practice and research purposes of fentanyl and doxapram. Fentanyl was quantified in 370 plasma samples from 92 preterm infants, and doxapram and keto-doxapram in 248 plasma samples from 65 preterms. The considerably high proportion of samples below LLOQ (21%

for fentanyl, 12% for doxapram, 13% for keto-doxapram) was due to the objective of the study on investigating drug pharmacokinetics. Namely, to estimate clearance of the investigated drugs opportunistic sample collection was allowed up to and beyond the time at which the plasma concentrations decreased below the LLOQ. All samples collected during continuous administration of both drugs were all above the LLOQ for all three analytes, and only above ULOQ for three doxapram samples shortly after a bolus administration. In conclusion, the assay performed well for samples in clinical practice. Furthermore, investigation is currently in progress in which this method has been applied to several pharmacokinetic studies in preterm born infants up to elderly patients.

Despite the good performance, our assay has certain limitations. First, the stability of cefazolin and keto-doxapram did not reach the desired 120 hours at 15°C in the autosampler. Although as performance of the assay is finished within 48 hours, this did not create a problem in practice. Second, although the small plasma volume of 50µL for performing the assay, this may be too much for some preterm infants, and for quantification of multiple drugs requiring the use of different assays. Third, the LLOQ of certain analytes in our assay was higher than some reported assays quantifying a single analyte. This is due to our goal to quantify multiple analytes in one run simultaneously, which makes it more difficult to achieve maximal performance for all analytes. Nevertheless, the LLOQs of our assay all meet the clinically required limits of quantification. Fourth, the assay did not include inactive metabolites as these are not relevant for clinical practice. Therefore, only keto-doxapram was included being the active metabolite of doxapram.

It has been suggested to implement therapeutic drug monitoring as a supportive tool for analgosedation for fentanyl and sufentanil which may help physicians increase patient comfort regarding intra- and inter-operative interventions (Nosseir, Michels, Binder, Wiesen & Muller, 2014). Also for Beta-lactam antibiotics the contributive value of TDM has been suggested (Goriainov, Cook, J, D & Oreffo). Quantification of doxapram and keto-doxapram during therapeutic dosages of doxapram may be relevant to improve successful therapy even further in treatment of apnea of prematurity (Hayakawa, Hakamada, Kuno, Nakashima & Miyachi, 1986), and for evaluation of safety (Barbe et al., 1999).

5. CONCLUSIONS

We have developed a method for the simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and keto-doxapram in 50µL human plasma within a runtime of only 5.0

minutes. This greatly facilitates further research into these drugs as well as possible TDM purposes, even in the smallest plasma volumes obtained from preterm infants.

Acknowledgements

The authors value highly the cooperation of all nurses, and employees of the Neonatal Intensive Care Units in the Netherlands participating to the DINO study; Erasmus Medical Center, Rotterdam; Radboudumc, Nijmegen; Máxima Medical Center, Veldhoven; Maastricht Medical Center, Maastricht.

Accepted Article

References

- Aranda J. V. B., K.; Rex, J.; Linder, N.; Blanchard, P. (1988). High Pressure Liquid Chromatographic Microassay for Simultaneous Measurement of Doxapram and its Metabolites in Premature Newborn Infants. *Journal of Liquid Chromatography*; 11 (14): 2983-2991.
- Barbe F., Hansen C., Badonnel Y., Legagneur H., Vert P. & Boutroy M. J. (1999). Severe side effects and drug plasma concentrations in preterm infants treated with doxapram. *Ther Drug Monit*; 21 (5): 547-52.
- Carlier M., Stove V., Roberts J. A., Van de Velde E., De Waele J. J. & Verstraete A. G. (2012). Quantification of seven beta-lactam antibiotics and two beta-lactamase inhibitors in human plasma using a validated UPLC-MS/MS method. *Int J Antimicrob Agents*; 40 (5): 416-22.
- Chanques G., Jaber S., Barbotte E., Violet S., Sebbane M., Perrigault P. F., ... Eledjam J. J. (2006). Impact of systematic evaluation of pain and agitation in an intensive care unit. *Crit Care Med*; 34 (6): 1691-9.
- Clavijo C. F., Thomas J. J., Cromie M., Schniedewind B., Hoffman K. L., Christians U. & Galinkin J. L. (2011). A low blood volume LC-MS/MS assay for the quantification of fentanyl and its major metabolites norfentanyl and despropionyl fentanyl in children. *J Sep Sci*; 34 (24): 3568-77.
- Coppini R., Simons S. H., Mugelli A. & Allegaert K. (2016). Clinical research in neonates and infants: Challenges and perspectives. *Pharmacol Res*; 108: 80-87.
- FDA (2003). Guidance for Industry—Bioanalytical Method Validation. *United States Department of Health and Human Services Food and Drug Administration*.
- Fernandez Mdel M., Wille S. M., Kummer N., Di Fazio V., Ruysinckx E. & Samyn N. (2013). Quantitative analysis of 26 opioids, cocaine, and their metabolites in human blood by ultra performance liquid chromatography-tandem mass spectrometry. *Ther Drug Monit*; 35 (4): 510-21.
- Flint R., Halbmeijer N., Meesters N., van Rosmalen J., Reiss I., van Dijk M. & Simons S. (2017a). Retrospective study shows that doxapram therapy avoided the need for endotracheal intubation in most premature neonates. *Acta Paediatr*; 106 (5): 733-739.
- Flint R., Weteringen W. V., Voller S., Poppe J. A., Koch B. C., Groot R., ... Simons S. H. (2017b). Big data analyses for continuous evaluation of pharmacotherapy: A proof of principle with doxapram in preterm infants. *Curr Pharm Des*.
- Goriainov V., Cook R., J M. L., D G. D. & Oreffo R. O. (2014). Bone and metal: an orthopaedic perspective on osseointegration of metals. *Acta Biomater*; 10 (10): 4043-57.
- Hayakawa F., Hakamada S., Kuno K., Nakashima T. & Miyachi Y. (1986). Doxapram in the treatment of idiopathic apnea of prematurity: desirable dosage and serum concentrations. *J Pediatr*; 109 (1): 138-40.
- Hisada T., Katoh M., Hitoshi K., Kondo Y., Fujioka M., Toyama Y., ... Nadai M. (2013). A simple liquid chromatography-tandem mass spectrometry method for determination of plasma fentanyl concentration in rats and patients with cancer pain. *Biol Pharm Bull*; 36 (3): 412-6.
- Kiriazopoulos E., Zaharaki S., Vonaparti A., Vourna P., Panteri-Petratou E., Gennimata D., ... Panderi I. (2017). Quantification of three beta-lactam antibiotics in breast milk and human plasma by hydrophilic interaction liquid chromatography/positive-ion electrospray ionization mass spectrometry. *Drug Test Anal*; 9 (7): 1062-1072.
- LeGatt D. F., Beaudry M. A. & Bradley J. M. (1986). Simultaneous determination of doxapram and 2-ketodoxapram in plasma of neonates by gas chromatography. *J Chromatogr*; 378 (2): 478-81.
- Lillico R., Sayre C. L., Sitar D. S., Davies N. M., Baron C. M. & Lakowski T. M. (2016). Quantification of cefazolin in serum and adipose tissue by ultra high performance liquid chromatography-Tandem mass spectrometry (UHPLC-MS/MS): application to a pilot study of obese women undergoing cesarean delivery. *J Chromatogr B Analyt Technol Biomed Life Sci*; 1031: 94-98.
- Mahlke N. S., Ziesenitz V., Mikus G. & Skopp G. (2014). Quantitative low-volume assay for simultaneous determination of fentanyl, norfentanyl, and minor metabolites in human plasma and urine by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Int J Legal Med*; 128 (5): 771-8.
- Mather L. E. (1983). Clinical pharmacokinetics of fentanyl and its newer derivatives. *Clin Pharmacokinet*; 8 (5): 422-46.

- Matuszewski B. K., Constanzer M. L. & Chavez-Eng C. M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem*; 75 (13): 3019-30.
- McWhinney B. C., Wallis S. C., Hillister T., Roberts J. A., Lipman J. & Ungerer J. P. (2010). Analysis of 12 beta-lactam antibiotics in human plasma by HPLC with ultraviolet detection. *J Chromatogr B Analyt Technol Biomed Life Sci*; 878 (22): 2039-43.
- Nichol H., Vine J., Thomas J. & Moore R. G. (1980). Quantitation of doxapram in blood, plasma and urine. *J Chromatogr*; 182 (2): 191-200.
- Nosseir N. S., Michels G., Binder P., Wiesen M. H. & Muller C. (2014). Simultaneous detection of ketamine, lorazepam, midazolam and sufentanil in human serum with liquid chromatography-tandem mass spectrometry for monitoring of analgesedation in critically ill patients. *J Chromatogr B Analyt Technol Biomed Life Sci*; 973C: 133-141.
- Pacifici G. M. (2015). Clinical pharmacology of fentanyl in preterm infants. A review. *Pediatr Neonatol*; 56 (3): 143-8.
- Palleschi L., Lucentini L., Ferretti E., Anastasi F., Amoroso M. & Draisci G. (2003). Quantitative determination of sufentanil in human plasma by liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal*; 32 (2): 329-36.
- Parker S. L., Guerra Valero Y. C., Roberts D. M., Lipman J., Roberts J. A. & Wallis S. C. (2016). Determination of Cefalothin and Cefazolin in Human Plasma, Urine and Peritoneal Dialysate by UHPLC-MS/MS: application to a pilot pharmacokinetic study in humans. *Biomed Chromatogr*; 30 (6): 872-9.
- Prins S. A., Pans S. J., van Weissenbruch M. M., Walther F. J. & Simons S. H. (2013). Doxapram use for apnoea of prematurity in neonatal intensive care. *Int J Pediatr*; 2013: 251047.
- Robson R. H. & Prescott L. F. (1977). Rapid gas-liquid chromatographic estimation of doxapram in plasma. *J Chromatogr*; 143 (5): 527-9.
- Saar E., Gerostamoulos D., Drummer O. H. & Beyer J. (2010). Identification and quantification of 30 antipsychotics in blood using LC-MS/MS. *J Mass Spectrom*; 45 (8): 915-25.
- Saari T. I., Fechner J., Ihmsen H., Schuttler J. & Jeleazcov C. (2012). Determination of total and unbound sufentanil in human plasma by ultrafiltration and LC-MS/MS: application to clinical pharmacokinetic study. *J Pharm Biomed Anal*; 66: 306-13.
- Suzuki Y., Kamada N., Ohno H., Abe Y., Endo T. & Kobayashi M. (2017). Development and validation of an LC-MS/MS method for simultaneously determining doxapram and keto-doxapram in human serum. *Bioanalysis*; 9 (6): 503-515.
- Touw D. J., Neef C., Thomson A. H. & Vinks A. A. (2005). Cost-effectiveness of therapeutic drug monitoring: a systematic review. *Ther Drug Monit*; 27 (1): 10-7.
- Van Eeckhaut A., Lanckmans K., Sarre S., Smolders I. & Michotte Y. (2009). Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects. *J Chromatogr B Analyt Technol Biomed Life Sci*; 877 (23): 2198-207.

Acce

TABLES

Table 1: Concentrations of all calibration standards and LLOQ standard

Analyte	LLOQ ($\mu\text{g L}^{-1}$)	S1 ($\mu\text{g L}^{-1}$)	S2 ($\mu\text{g L}^{-1}$)	S3 ($\mu\text{g L}^{-1}$)	S4 ($\mu\text{g L}^{-1}$)	S5 ($\mu\text{g L}^{-1}$)	S6 ($\mu\text{g L}^{-1}$)	S7 ($\mu\text{g L}^{-1}$)	S8 ($\mu\text{g L}^{-1}$)
Fentanyl	0.10	0.10	0.50	1.0	2.5	4.0	5.0	8.0	10.0
Sufentanil	0.25	1.0	5.0	10.0	25.0	40.0	50.0	80.0	100.0
Cefazolin	1,000	1,000	5,000	1,0000	25,000	75,000	80,000	90,000	100,000
Doxapram	50	100	500	1,000	2,500	4,000	5,000	8,000	10,000
Keto-doxapram	50	50	100	250	500	1,000	2,000	4,000	5,000

S: Calibration standard - LLOQ: Lower limit of quantification

Accepted Article

Table 2: Concentrations of all quality controls

Analyte	QC Low ($\mu\text{g L}^{-1}$)	QC Medium ($\mu\text{g L}^{-1}$)	QC High ($\mu\text{g L}^{-1}$)
Fentanyl	0.5	2.5	7.5
Sufentanil	2.0	10.0	30
Cefazolin	4,000	25,000	70,000
Doxapram	400	2,500	7,000
Keto-doxapram	150	850	3,000

QC: Quality control

Accepted Article

Table 3: MS/MS settings

Analyte	Parent ion (m/z)	Product ion (m/z)	Collision Energy (V)	S-Lens (V)
Fentanyl	337.400	188.200	22	124
Fentanyl-d5	342.400	188.200	22	124
Sufentanil	387.300	238.200	18	124
Cefazolin	455.050	323.00	10	80
Doxapram	379.250	128.100	55	135
Keto-doxapram	393.241	214.100	26	150

Accepted Article

Table 4. Validation results (n=6)

Analyte	QC	Accuracy RSD (%)	Repeatability (within-run precision)			Reproducibility (between-run precision)			LLOQ ($\mu\text{g L}^{-1}$)	ULOQ ($\mu\text{g L}^{-1}$)
			Mean ($\mu\text{g L}^{-1}$)	SD ($\mu\text{g L}^{-1}$)	RSD (%)	Mean ($\mu\text{g L}^{-1}$)	SD ($\mu\text{g L}^{-1}$)	RSD (%)		
Fentanyl	L	-2.7	0.46	0.01	1.5	0.51	0.02	3.9	0.10	10.0
	M	-2.7	2.1	0.05	2.4	2.00	0.03	1.5		
	H	-3.4	6.8	0.09	1.3	6.82	0.15	2.2		
Sufentanil	L	3.0	5.7	0.06	1.0	5.60	0.14	2.5	0.25	50.0
	M	-0.2	26.7	0.44	1.7	25.5	0.56	2.2		
	H	-1.8	53.1	1.03	1.9	52.5	1.47	2.8		
Cefazolin	L	8.0	6.6	0.12	1.7	6.57	0.23	3.5	1,000	100,000
	M	0.4	32.1	0.39	1.2	30.9	0.34	1.1		
	H	0.9	101.6	1.48	1.5	104.2	1.25	1.2		
Doxapram	L	3.2	0.42	0.01	1.4	0.43	0.02	4.7	50	4,500
	M	3.0	2.15	0.04	1.9	2.22	0.02	0.9		
	H	-1.2	3.46	0.06	1.8	3.57	0.05	1.4		
Keto- doxapram	L	-4.8	0.16	0.00	1.5	0.18	0.01	5.7	50	5,000
	M	3.2	0.88	0.02	2.1	0.77	0.02	2.6		
	H	1.7	3.43	0.08	2.4	3.43	0.12	3.5		

Abbreviations: QC: Quality control - L: Low - M: Medium - H: High – SD: Standard deviation
– RSD: Relative standard deviation - LLOQ: Lower limit of quantification - ULOQ: Upper
limit of quantification

Table 5. Matrix effect, recovery and process efficiency

Analyte	Matrix effect mean (%)	Recovery mean (%)	Process Efficiency mean (%)
Fentanyl	113.3	102.2	115.9
Sufentanil	108.8	93.5	101.7
Cefazolin	108.0	90.1	97.4
Doxapram	111.3	92.5	102.9
Keto-doxapram	99.9	99.2	99.1

Accepted Article

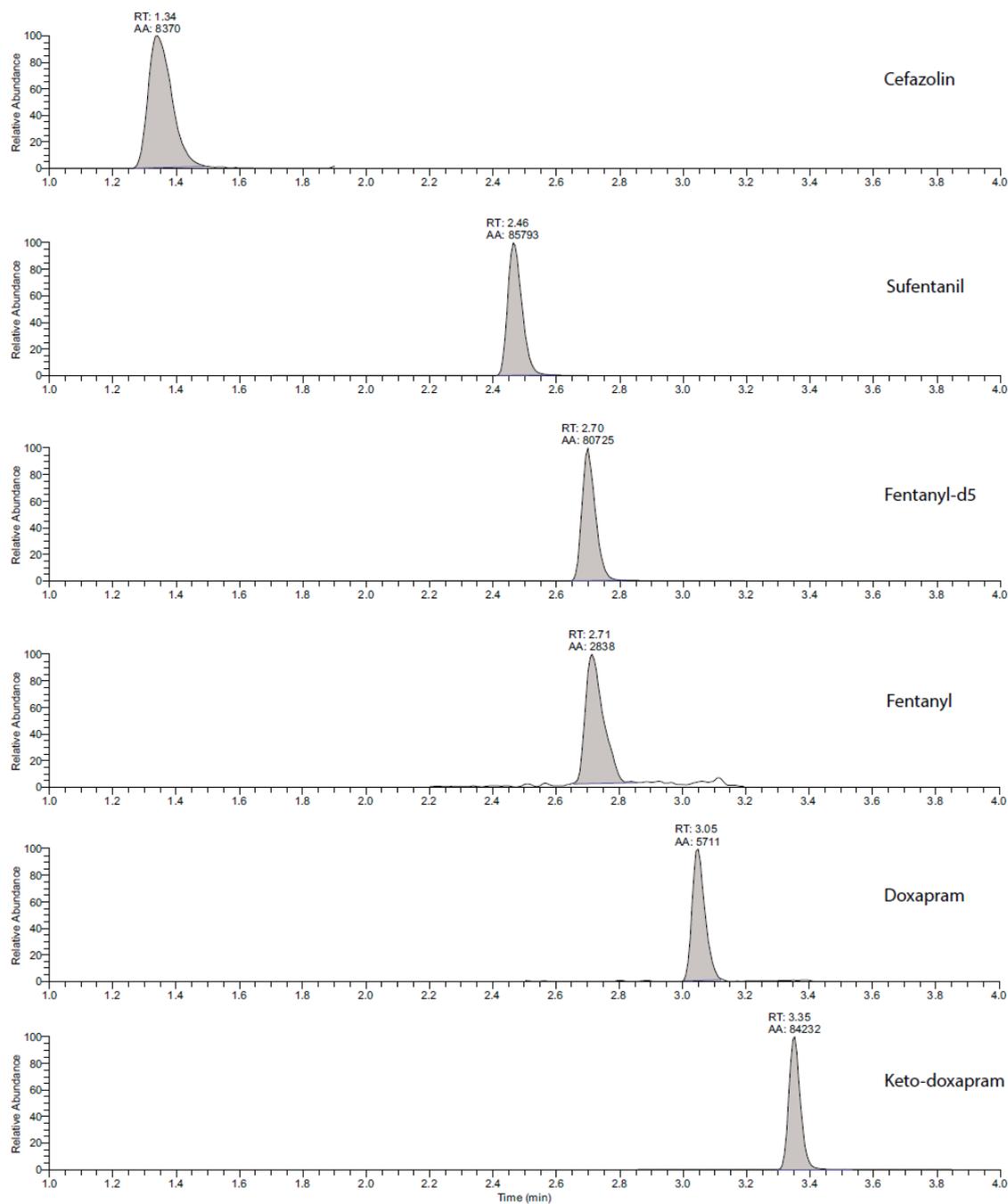


Figure 1. Ion chromatograms of all analytes and internal standard in lowest concentration calibration standard 1 (see Table 1)

For the ion chromatograms of sufentanil and fentanyl 10 μ L was injected, and for cefazolin, doxapram and keto-doxapram the injection volume was 1 μ L.

Abbreviations: RT: Retention time - AA: Automatic integrated area