A sensitive liquid chromatography method for analysis of propofol in small volumes of neonatal blood

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Figure 1. Chromatograms of propofol in a spiked blood sample at concentration of 10 $\mu\text{g/ml}$ in blood (a) and a blank blood sample (b).

	Method	Sample type	Sample volume (ml)	Sample preparation	LOQ (ng/ml)	Reference No.
1	LC-UV	serum	0.45	SPE	20	[6]
2	LC-Fluo	blood/plasma	0.6	LLE	100	[7]
3	LC-Fluo	blood	0.4	LLE	2	[8]
4	LC-Fluo	plasma	0.5	PP	3	[9]
5	LC-MS/MS	plasma	0.5	SPE	10	[11]
6	LC-MS/MS	plasma	1	SPE	5	[10]
7	LC-MS/MS	plasma	0.05	DE	13	[12]

Table 1. Existing HPLC methods for propofol determination in human blood, plasma and serum samples.
LLE, liquid-liquid extraction; SPE, solid phase extraction; PP, protein precipitation; DE, derivatization.

Propofol intraday assay (n=6)			
Concentration (µg/ml)	Concentration measured (µg/ml)	Accuracy (bias, %)	Precision (RSD, %)
0.05	0.056	13.5	1.3
0.5	0.48	-4.3	0.2
5	4.8	-4.8	0.2
Propofol interday assay (n=4)			
0.05	0.054	7.7	5.2
0.5	0.50	0.8	5.7
5	5.1	1.4	5.8

Table 2. Intraday and interday assay precision and accuracy for propofol in blood.

Figure 2. Box-Plot of the maximum, median, 1st and 3rd quartiles and minimum of the measured propofol concentrations at 3h and 12h from 50 neonates.

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ABSTRACT

What is known and objective:

Sampling volume of blood from neonates is limited due to small total blood volume. However, most of the published propofol analysis assays require a relatively large blood sample volume (typically ≥ 0.5 ml). Therefore, the aim of the present study was to develop and validate a sensitive method requiring a smaller sample volume (0.2 ml) to fulfill clinical relevant research requirements.

Methods:

Following simple protein precipitation and centrifugation, the supernatant was injected into the HPLC-fluorescence system and separated with a reverse phase column. Propofol and the internal standard (thymol) were detected and quantified using fluorescence at excitation and emission wavelengths of 270 nm and 310 nm, respectively. The method was validated with reference to the Food and Drug Administration (FDA) guidance for industry. Accuracy (CV, %) and precision (RSD, %) were evaluated at three quality control concentration levels (0.05, 0.5, 5 $\mu\text{g/ml}$).

Results and discussion:

Calibration curves were linear in the range of 0.005–20 $\mu\text{g/ml}$. Intra- and interday accuracy (–4.4%–13.6%) and precision (0.2%–5.8%) for propofol were below 15%. The calculated LOD (limit of detection) and LLOQ (lower limit of quantification) were 0.0021 $\mu\text{g/ml}$ and 0.0069 $\mu\text{g/ml}$, respectively. Propofol samples were stable for 4 months at -20°C after the sample preparation. This method was applied for analyzing blood samples from 50 neonates that received propofol, as part of a dose-finding study. The measured median (range) concentration was 0.14 (0.01–1.11) $\mu\text{g/ml}$, which were in the range of the calibration curve. The calculated median (range) propofol half-life of the gamma elimination phase was 10.2 (4.7–26.7) h.

What is new and conclusion:

A minimal volume (0.2 ml) of blood samples from neonates required for the determination of propofol with the analyses method we developed. The method can be used to support the quantification of

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propofol drug concentrations for pharmacokinetic studies in the neonatal population and to avoid large blood sampling.

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1. What is known and objective

Propofol (2,6-diisopropylphenol) is widely used as an intravenous agent for induction and maintenance of anesthesia [1]. Propofol has a high metabolic clearance and is rapidly eliminated by either glucuronidation (main route) or hydroxylation (minor route) [2]. Induction with propofol is fast and smooth and rapidly followed by complete recovery with a low incidence of post-operation nausea and vomiting [3].

There are a number of reports on the quantification of propofol in blood or plasma using HPLC coupled with either UV [4-6], fluorescence [7-9] or MS/MS [10-12] (**Table 1**). Some of the HPLC-MS/MS methods are more sensitive compared to UV and fluorescence detection, however, they are costly and may not be available in every (medical/clinical) laboratory. Most of the published propofol HPLC assays require a relatively large blood volume (typically ≥ 0.4 ml [8]) to generate reliable results. The need for a large sample volume significantly reduces the feasibility of pediatric pharmacokinetic (PK) studies. The problem is most apparent in preterm newborns where ethical and practical considerations prohibit large volume sampling. In premature neonates, blood sampling is especially limited due to the very small volume of circulating blood. As a general rule, the blood drawn for laboratory testing should not exceed 5% of total blood volume (80 ml/kg to 115 ml/kg for neonates) per 24h [13]. It was reported that in very low weight neonates, up to 2.4% of calculated circulating blood volume (2.3 ml/kg) of blood can be drawn for scientific purposes without changing the basic hemodynamic parameters, Hgb and Ht values, blood component transfusions or fluid requirements [14].

In PK studies, it is important to measure the concentrations accurately and precisely. Although the PK of propofol in adults is well documented, propofol exposure data in neonates are very limited [15, 16]. Whole blood is the preferred sample matrix for propofol analysis since it is significantly bound to serum proteins (48%, predominantly) and erythrocytes (50%) [17, 18]. The blood: plasma ratio for propofol was found to be 1.13 while the calculated blood-cell: plasma ratio was 1.28 [19, 20], indicating that propofol is mostly concentrated in blood cells. Furthermore, whole blood concentrations describe the total drug content in the circulatory system, while allowing to convert between plasma and blood when

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necessary. Thymol is the most frequently used internal standard in published researches for the analysis of propofol besides eugenol [21] and 2,4-ditert-butylphenol [8] which were also used in some methods. In the present study, a selective and sensitive HPLC-fluorescence method for the analysis of blood samples containing propofol was developed and validated. The method allowed to reduce the sample volume to 0.2ml which enabled reliable quantitative determination of propofol in clinical blood samples obtained from neonates.

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2. Methods

2.1. Chemicals and standards

All chemicals used were HPLC grade. Propofol ($\geq 98.0\%$) for method development and validation was purchased from TCI Europe (Antwerp, Belgium), while Diprivan[®] (10 mg/ml, AstraZeneca, Brussels, Belgium) was used for clinical propofol administration, as reported earlier[16]. Thymol ($\geq 99.5\%$) was purchased from Sigma-Aldrich (Belgium). Formic acid ($\geq 99.0\%$, ULC/MS grade) was obtained from Biosolve B.V. (The Netherlands). Methanol and acetonitrile (99.0%, HPLC grade) were purchased from Acros Organics (Belgium). Stock solutions of propofol (2 mg/ml) and thymol (1 mg/ml) were prepared in methanol and stored at -20°C .

2.2. Chromatographic apparatus and conditions

The HPLC-system consisted of a Waters 600E pump, combined with a Waters 717plus autosampler and a Waters 2475 multi-wavelength fluorescence detector. The excitation and emission wavelengths were set at 270 and 310 nm, respectively. The injection volume was 35 μl . Chromatographic separation of propofol and thymol was performed over a total run time of 12 min on a Gemini[®] C18 column (3 μm , 4.6 mm x 150 mm, Phenomenex, Utrecht, The Netherlands) protected with a Gemini[®] C18 SecurityGuard[®] cartridge (3 μm , 4 mm x 3 mm, Phenomenex, Utrecht, The Netherlands) both maintained at 30°C . The mobile phase consisted of a mixture of acetonitrile and water containing 0.1% v/v formic acid (70:30, v/v) which was delivered to the system isocratically at a flow rate of 0.6 ml/min.

2.3. Sample preparation

To 1 volume of whole blood sample, 0.1 volume of the internal standard (thymol, 5 $\mu\text{g}/\text{ml}$ in 50:50 methanol:water) and 2 volumes of acetonitrile were added. Subsequently, samples were vortexed 2 times for 15 s before being centrifuged ($20,816 \times g$) at 4°C for 10 min. The supernatant was transferred to a new recipient and stored at -20°C until analysis. At the day of analysis, the supernatant was thawed,

vortexed and centrifuged (20,816 g) at 4°C for 10 min. 150 µl of each supernatant was transferred into a micro-insert for HPLC-vials and injected directly into the HPLC-system.

2.4. Calibration standards and quality controls

Calibration curves of propofol were constructed by preparing 10X concentrated dilution series in a mixture of methanol: water (50:50, v/v). For each concentration, 20 µl of the 10X concentrated solution was transferred to 180 µl of blank whole blood, after which they were processed in the same manner as whole blood samples with a range of 0.005-20 µg/ml. Final concentrations were 0.005, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 20 µg/ml. Quality control samples were prepared at concentrations of 0.05, 0.5 and 5 µg/ml, processed and stored at -20°C.

2.5. Limit of detection (LOD) and lower limit of quantification (LLOQ)

The LOD was calculated as the lowest concentration that the bioanalytical procedure can reliably differentiate from background noise and the LLOQ was the lowest concentration that can be quantitatively determined with acceptable precision and accuracy according to the FDA [22]. LOD was determined based on the standard deviation of the response (S_y) of the curve and the slope of the calibration curve (S). The LOD was at levels approximating the LOD according to the formula: $LOD = 3.3 (S_y/S)$. The standard deviation of the response was determined based on the standard deviation of the y-intercept of the regression line. For the LLOQ, the calculation method is again based on the S_y and S according to the formula: $LLOQ = 10 (S_y/S)$.

2.6. Recovery, Precision and Accuracy

The precipitation recovery was determined by calculating the ratio (%) of the area response (normalised for internal standard) of samples after precipitation with the pure standard solutions at QC concentrations.

Accuracy was determined by replicates analyses of high, middle and low concentrations (0.05, 0.5 and 5 µg/ml) to meet the FDA guidance for bioanalytical method validation [22]. The deviation of the mean from the true value should be within 15% of the actual value except at LLOQ, where it should not deviate

by more than 20%. The precision of the assay was measured as the percent coefficient of variation over the high, middle and low concentrations respectively during the course of validation. Intraday assay variations were determined by analyzing six replicate samples at each concentration on a single day. Interday assay variations were determined by analyzing four replicate samples at each concentration on four separate days.

2.7. Stability

A series of blood samples spiked with propofol at concentrations of 0.05, 0.5 and 5 µg/ml were prepared. Blood samples proceeded with sample preparation and the final samples were stored at -20 °C for 4 months. Five replicate samples were analyzed for each concentration. **Stability of propofol at three QC samples was studied for 24h at 4 °C to mimic the autosampler conditions.** The stability of propofol was determined by comparing the peak areas obtained from stored samples and freshly prepared samples. Samples were considered stable if the differences in peak area between the stored and freshly prepared samples were ≤15% [20].

2.8. Application

This method was developed to enable a propofol dose-finding study in neonates[16]. The clinical study was registered (ClinicalTrials.gov: NCT01621373; EudraCT: 2012-002648-26) and was approved by the ethical board of the University Hospitals-Leuven. Patients were eligible for inclusion when following criteria were met: prescription of propofol (IV bolus, immediately followed by NaCl 0.9% 1 ml/kg in 30 seconds) for procedural sedation to facilitate (semi-)elective endotracheal intubation. Patients were only recruited following written informed consent of the parents. The initial individual propofol dose administered was determined using a dose-finding approach as described previously, with subsequent up-titration of the dose based on clinical need. The initial and total propofol bolus dose ranges used throughout the study were 0.5-2 mg/kg and 0.5-4.5 mg/kg respectively [16]. The developed methodology was applied to a series of blood samples collected from 50 included neonates receiving propofol for the procedural sedation. Blood samples were collected from an arterial line if available, or by venous puncture when sampling for medical reasons was necessary, at 3h and 12h after propofol

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administration. Blood samples (300-600 µL/sample) were collected in oxalate tubes (BD Vacutainer) and a maximum total blood volume of 1 ml/kg collected in each individual neonate was respected. Blood samples were stored at 4°C, for a maximum period of 4 weeks, until processing.

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3. Results and discussion

3.1. Blood sampling volume from neonates

In this study, only 0.2 ml of blood was needed for sample preparation and analysis. Most of the published assays require a larger blood volume (typically > 0.5 ml) [8, 9] (**Table 1**) to generate a practical analysis. This prerequisite significantly limits the feasibility of pediatric PK studies and the problem is most likely in preterm newborns where large volume sampling is prohibited by reason of ethical considerations. The European Medicines Agency (EMA) recommended a maximum of 1 % of blood loss (the total average blood volume was 109 ml per kilogram for preterm neonates at birth [23]) on each occasion and 3 % over 4 weeks [24].

3.2. Sample treatment

High precipitation recovery ($103.31 \pm 4.80\%$ (average, RSD)) was observed after sample preparation compared to freshly prepared propofol in solvents. Bioanalysis of whole blood samples is a challenge due to the complex matrix and the presence of erythrocytes. The pre-treatment procedure is needed to simplify the blood matrix before analysis and should produce a high degree of recovery for the analytes in the sample. Precipitation is widely used for whole blood and plasma samples to remove the protein and organic solvents [25]. Acetonitrile was found to be a superior organic precipitant in a ratio of 2:1 with whole blood [26]. For standardization purposes, two times vortex (each for 30 seconds) with a one-minute interval in between was applied for all the samples after adding the acetonitrile. The second vortex was found definitely necessary for repeatable results and high recovery. **Sample preparation using protein precipitation is easily repeatable and straight-forward. The current method only needs a limited volume (0.4 mL) of organic solvents, which is lower than amounts used for LLE methods [8]. In addition, this procedure avoids the use of a costly and labour-intensive SPE methods.**

3.3. Method Validation

Specificity testing was performed with blank blood samples from neonates, which were prepared in the same way as the study samples. The endogenous components did not interfere with the compounds of interest. **Figure 1** shows chromatograms of a blank blood sample (b) and a blood sample spiked with propofol at a concentration of 10 µg/ml (a). Retention times were approximately 8.2 min and 5.8 min for propofol and the internal standard thymol, respectively. The total run time for each sample was 12 min.

The standard curves for propofol were linear in the (0.005 µg/ml – 20 µg/ml) concentration range with a correlation coefficient of at least 0.9991. Satisfactory results were obtained for both intra and interday assays, which is shown in **Table 2**. The precision and accuracy were less than 15% at all concentration points including the LLOQ, which was within the allowable range.

The high recoveries of propofol and thymol indicate analyte stability under the extraction conditions applied and high extraction efficiency. Consistent recovery values at QC concentrations indicate that the sample preparation process is equivalent across this range.

The LOD was 0.0021 µg/ml while the LLOQ was 0.0069 µg/ml. This method has a higher sensitivity (LLOQ = 0.0069 µg/ml) than the published LC-UV method[6] (LOQ = 0.02 µg/ml). **Yarbrough et al. reported an LC-UV method with LLOQ at 5 ng/ml. However, the sample volume needed for analysis was twice the volume as compared to the volume used in our method.** The presently developed HPLC-Fluo method has a comparable to a slightly better sensitivity than the LC-MS/MS methods (LLOQ = 0.005 µg/ml [10] and 0.013 µg/ml [12]). Moreover, MS detectors are more expensive and may not be readily accessible in many laboratories.

Propofol in whole blood was suggested to be stored at 4°C instead of -20°C because of significant propofol loss when it's frozen[27]. It was reported that propofol in blood was stable for 12 weeks at 4°C[28]. Final samples were stable for 4 months at -20 °C. The stability results indicate that propofol samples were stable during the sample preparation process and final sample storage. The remaining percentages of QC samples stored at -20°C for 4 months compared with freshly prepared samples were

97.16±2.60 % (average, RSD). No degradation was observed for QC samples at 4°C for 24h. The recovery (value in stability sample/value in reference) was 99.80 ± 0.11% (average, RSD), indicating propofol was stable in the autosampler over 24h.

3.4. Application to blood samples from neonates

The method validated in this study was applied in blood samples taken from 50 neonates who received propofol for the procedural sedation. Summary statistics of the propofol blood concentrations at 3h and 12h after IV bolus administration (n=50 neonates) are depicted in the boxplot in **Figure 2**. The actual concentrations and the calculated half-life ($T_{1/2}$) of the gamma phase of the elimination are provided in the supplementary table. Based on our previous research [29], the both sampling times were in the gamma phase of propofol elimination. The calculated median half-life of the gamma elimination phase for those neonates was 10.2 (range 4.7-26.7) h. Measured concentrations, ranging from 0.01 µg/ml to 1.11 µg/ml, were within the range of the calibration curve, suggesting that the current method can be used successfully for the measurement of propofol levels observed in neonates. In addition, the concentration time data will be used to develop a PBPK (physiologically based pharmacokinetic) model that will be applied for a dose optimization study.

3.5. Advantages and disadvantages

The presently developed method uses acetonitrile precipitation and is straight-forward without using a large amount of organic solvent like in the case of an LLE method. Furthermore, this easily repeatable method avoids the use of costly SPE columns. This method can be applied widely since HPLC-Fluo instruments are available in most bioanalytical labs. The high sensitivity made it possible to determine the samples at a later sampling time (12h), in which low concentrations were expected. Moreover, the low blood sample volume requirement of the method supported the unique clinical/pharmacokinetic research in neonates.

What is new and conclusion

In conclusion, we successfully developed and validated an HPLC method with fluorescence detection for quantitative determination of propofol in a minimal volume (0.2 ml) of blood samples from neonates. The developed method is precise, accurate and sensitive and was applied to determine propofol blood concentrations in 50 neonates. The low volume of sample needed for analysis is especially applicable for the neonates who have a limited volume of blood. Overall, this propofol quantification method can be widely used in the pediatric propofol sample analysis and related PK studies.

Conflict of interest

The authors declared no conflict of interest.

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