Quantification of Propofol in Small Volumes of Plasma using High-performance Liquid Chromatography with Fluorescence Detection. Therapeutic Drug monitoring of one Patient during and after Infusion in the Perioperative Period of Cardiac Surgery

Carlos R. DA SILVA FILHO 1, Roberto T.O. KONDO 1, Andréia Z. CHIGNALIA 1, Karin V. LÓPEZ 1, Valeria A. PEREIRA 1, Maria J.C. CARMONA 2, José O.C. Auler JUNIOR 2 & Silvia R.C.J. SANTOS 1*

1 Department of Pharmacy, School of Pharmaceutical Sciences of University of Sao Paulo, Av. Prof Lineu Prestes, 580, São Paulo, SP, Brazil.
2 Instituto do Coracao HCFMUSP, Av. Dr Enéas de Carvalho Aguiar, 44, São Paulo, SP, Brazil.

SUMMARY. A liquid chromatographic method with fluorescence detection for determination of propofol in small volumes of plasma has been adapted to the conditions of the laboratory using a C18 column. Peaks were eluted at 21 min propofol and at 12 min thymol (internal standard) using a binary mobile phase at flow rate 0.8 mL/min. The method validated presents 0.05 mg/L sensitivity, 0.1 - 10 mg/L linearity, \( r^2 0.9977 \), systematic error of 8.2 and 7.0% (intra- and interday accuracy), intra- and interday precision (CV %: 8.4% and 8.8%). Recovery 93.9%, good stability and robustness were reported. The analytical method was validated by the quantification of propofol in arterial plasma \( C_{\text{max}}:3.3 \text{ mg/L} \ C_{\text{min}}:0.2 \text{mg/L} \) of one patient received 1570 mg infusion of propofol in the perioperative period of surgery of myocardial revascularization. Depth of anaesthesia expressed as bispectral index (BIS) was 36 (32-40) in that period up to the 4th h, and an adequate hypnosis was obtained until the end of surgery.

INTRODUCTION

Propofol is a short-acting hypnotic drug that is widely used in both ambulatory and hospitalised patients in minor and in major surgeries using the target controlled infusion (TCA) to reach 0.5-1.0 mg/L or 3-4mg/L, respectively. Because it permits the efficient control of anaesthetic depth, a rapid and controllable recovery, propofol may be useful in the titration of anaesthetic depth, including postoperative sedation of cardiac surgical patients. It is administered by a target controlled infusion (TCA), a system developed to reach and maintain the desired plasma concentrations of drugs using averaged pharmacokinetic models derived from population samples. The system allows anaesthetic drug to be administered to a theoretical target plasma concentration calculated mathematically by the delivery system algorithm 1.

The analysis of plasma concentrations of propofol aids in attempts to determine the minimal dose sufficient for an individual patient to maintain anaesthesia and to help the decrease of risk on drug-related complications and interactions. Such studies require highly sensitive, specific and reproducible techniques. High-performance liquid chromatography is the most frequently analytical technique applied for deter-

KEY WORDS: Heart surgery, CLAE-F, Propofol, Therapeutic drug monitoring.

PALABRAS CLAVE: Cardiocirugía, HPLC-F, Monitoreo terapéutico, Propofol.

* Autor a quem correspondência deve ser enviada. E-mail: pharther@usp.br
mining the concentration of drug in body fluids using ultraviolet, electrochemical or fluorimetric detectors 2-14.

Ultraviolet detection is not so selective, while electrochemical device is the most expensive. The combination of HPLC and fluorescence detection has improved the selectivity of propofol measurements. Additionally, the clean-up of biological samples is essential in conventional analysis before determination by HPLC. Precipitation of plasma using acetonitrile or drug extraction with organic solvents have the advantage of low costs instead solid phase cartridges 9-10. Analytical procedures reported previously applying HPLC-F showed some disadvantages like high flow rate, and narrow range of linearity.

Analytical methods reported in the literature employ complex mobile phases at a high flow rate for the chromatographic separation, in addition to laborious and time-consuming procedures such as liquid-liquid extraction or expensive techniques as solid-phase extraction for the purification of plasma samples. Most methods require 0.5 to 1 mL aliquots of the biological sample that represent an ethical limitation for the execution of clinical trials which generally require a large number of blood sample collections. Therefore, the objective of this study was to apply a simple, rapid, selective and sensitive analytical method adapted from previous procedures for the quantification of propofol by HPLC using small plasma volumes. The present method was also validated by the therapeutic monitoring of one patient received propofol during and after the drug infusion.

MATERIAL AND METHODS

Chemicals

HPLC grade methanol and acetonitrile used in the analytical procedure were purchased from Merck (Darmstadt, Germany). Acetic acid used for water acidification in mobile phase preparation was analytical grade (Merck, Darmstadt, Germany). Thymol (internal standard) was purchased from Sigma Aldrich (St Louis MO, USA). Propofol was generously obtained from AstraZeneca (Detroit, USA). Purified water was obtained from Simplicity System (Millipore Corporation Bedford MA, USA).

Thymol (stock solution) was prepared by dissolving the drug in methanol and diluting to 1 mg/mL, stored at -20 °C. The solution was appropriately diluted with blank plasma tested previously, to prepare plasma standards and to obtain the following concentrations for the calibration curve prepared everyday: 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 mg/L and the internal controls (0.4, 2.0 and 4.0 mg/L); all sample controls were stored at -20 °C. Both propofol stock solution, plasma standards and internal controls were shown to be stable up to 12 months.

Instrumentation

The chromatographic system consisted of a Shimadzu model LC-10AVP solvent delivery module (Shimadzu, Kyoto, Japan), equipped with a Shimadzu autosampler model SIL-10AD-VP and a fluorescence detector model RF10AXL (Shimadzu, Kyoto, Japan). The system was controlled by the software Class VP version 5.03, Shimadzu (Kyoto, Japan). The analytical column was a Shim-Pack CLC - ODS C18™ Shimadzu (Kyoto, Japan), 150 X 6.0 mm, particle size 5 µm, adapted to a guard-column C18 Waters (Milford, USA).

HPLC Conditions

The mobile phase consisted of a mixture of acetonitrile and acidified water pH: 4.6 (60:40, v/v); acidified water was obtained by the addition of acetic acid 1.2% (v/v) to purified water. The mobile phase was freshly prepared on the day of use and helium degassed; the chromatographic analysis was performed in an isocratic system of elution using a flow rate of 0.8 mL/min at room temperature. The excitation of the fluorescence detector was set to 276 nm; the emission was recorded at 310 nm. The run time of 25 minutes guaranteed the selectivity of chromatographic analysis and 50 samples in a rack of the autosampler were injected daily in a volume of injection equivalent to 0.05 mL.

Analytical Procedure

Clean-up of calibration standards and clinical samples

Aliquots of methanolic working solution of internal standard, 5 mg/L were added (0.05 mL containing 250 ng thymol/assay) to a Eppendorf tube (1.2 mL), methanol was evaporated to dryness in a stream of purified nitrogen at 37 °C followed by addition of 0.2 mL of plasma and 0.6 mL of acetonitrile to precipitate the plasma proteins. Mixture was vortexed for 30 seconds, centrifuged at 6000 rpm for 45 minutes at 5 °C.
Supernatant (0.3 mL) was transferred to a conic glass tube and the organic solvent was concentrated to dryness in a stream of purified nitrogen at 37 °C. Residue was dissolved with 0.1 mL of mobile phase and aliquots of 0.05 mL were injected into HPLC in the chromatographic conditions above detailed.

Samples obtained from a patient received propofol by infusion were centrifuged at 1500 g for 30 minutes and plasma was stored at -20 °C prior to analysis. Patient samples were purified by the same procedure described for the calibration standards and internal controls. The concentration of propofol in the samples was calculated with reference to a calibration curve generated from sample standards analysed along with each batch of clinical samples. Only 0.2 mL of plasma was required for the assay.

Linear least-squares regression was performed on the peak-area ratio (analyte peak area / internal standard peak area) versus nominal concentration values. Linearity and validation studies were performed based on 7 calibration standards of propofol (0.1 to 10.0 mg/L). A calibration day curve (0.1-5.0 mg/L) was constructed for propofol in plasma. Propofol plasma concentrations in the samples were determined from their peak-area ratios calculated by the standard curve equation.

Analytical method was developed on the basis of confidence parameters as linearity, limit of quantification (LOQ, n=10) and limit of detection (LOD, n=10), accuracy and precision (within and inter-day), absolute recovery, robustness and stability studies. Linearity study (0.1 to 10.0 mg/L) required 7 calibrators, while 6 calibrators (0.1-5.0 mg/L) were required to construct the day calibration curve. In parallel, three spiked blank plasma (internal controls: 0.4, 4.0 and 4.0 mg/L, duplicate) containing propofol were analysed every day. Peak area ratio, propofol to internal standard, was plotted against propofol plasma concentration to obtain the calibration curve with six points showing linear correlation coefficient: \( r^2 > 0.99 \). The day curve was accepted, if at least 4/6 of the internal controls presenting systematic error lower than 15%; the two possible rejected controls never can be the replicate. The limit of detection was determined at signal noise ratio of 3, while the limit of quantification was the lowest concentration determined by the analytical method with precision lower than 15%.

A calibration curve of 7 concentrations (0.1 to 10 mg/L) was applied to linearity study and also to estimate the absolute recovery of propofol from biological matrix, using three different concentrations, 0.4, 4.0 and 8.0 mg/L (3 replicates/each). The same calibration curve of 7 concentrations (0.1 to 10.0 mg/L, triplicates) was applied to estimate intra and inter-day precision of propofol from biological matrix, using three different concentrations, 0.4, 4.0 and 8.0 mg/L (n = 6 replicates/each) during three consecutive days. Data were expressed as coefficient of variation, percentage. Similarly, a calibration curve of 7 concentrations (0.1 to 10.0 mg/L) was applied to estimate within-day and inter-day accuracy of propofol from biological matrix, using 3 different concentrations, 0.4, 4.0 and 8.0 mg/L (n = 6 replicates/each) during 3 consecutive days. Data were expressed by systematic error as percentage.

**Stability Studies**

The stability of propofol in plasma was tested in a diverse manner as follows: at room temperature in the autosampler, a serial of microvials containing propofol in plasma extract submitted to HPLC analysis was tested for 24 hours for the stability of drug dissolved in the mobile phase, against the day calibration curve and the respective internal controls.

**Thawing cycles**

Biological matrices spiked blank plasma, using three different concentrations, 0.4, 4.0 and 8.0 mg/L (3 replicates/each) during three consecutive periods in the same day were analysed by HPLC after the clean-up of plasma samples as detailed above, to estimate the stability of drug in the biological matrix after three thawing cycles against to the day calibration curve and the respective internal controls. Data were expressed by systematic error as percentage.

At room temperature on the work-bench, a serial of plasma containing propofol, using 3 different concentrations, 0.4, 4.0 and 8.0 mg/L (n=3 replicates/each) were kept on the work-bench for 6 hours, then the clean-up of biological samples was performed followed by HPLC analysis, as detailed above, in parallel to the day calibration curve and the respective internal controls. Data were expressed by systematic error as percentage.

**Long-term stability**

A serial of plasma containing propofol, using 3 different concentrations, 0.4, 4.0 and 8.0 mg/L (3 replicates/each) were stored at -20 °C in the freezer for 12 months, then the clean-up of biological samples was performed followed by
HPLC analysis, as detailed above, in parallel to the day calibration curve and the respective internal controls. Data were expressed by systematic error as percentage.

**Robustness**

Was evaluated step by step using two different Shim-Pack C18 columns, little changes in the proportion of acetonitrile in the mobile phase and also in the flow rate. The study was developed using three different concentrations, 0.4, 4.0 and 8.0 mg/L (three replicates/each); the clean-up of biological samples was performed followed by HPLC analysis, as detailed above, in parallel to the day calibration curve and the respective internal controls. Data were expressed by systematic error as percentage.

**Propofol plasma monitoring in one patient submitted to cardiac surgery**

This method was applied to assay propofol concentrations in plasma samples of a cardiac patient, undergoing coronary artery bypass grafting (CABG) surgery, who received propofol intravenously. Patient, male, 73 years, 81 kg (body index: BI), 1.69 m (height), 1.94 m² (body surface area: BSA), 28.42 kg/m² (body mass index: BMI), with coronary insufficiency submitted myocardial revascularization. Propofol was utilised for the induction and the maintenance of anaesthesia during surgical intervention and drug was administered as a target controlled infusion 1-4 mg/L during the cardiac surgery. The protocol of the study was approved by the hospital’s Medical Ethics Committee (Heart Institute) and Ethics Committee of School of Pharmaceutical Sciences, University Sao Paulo. Patient was informed in details about the study procedures, including blood sampling, whose provided the informed, written consent to participate of the clinical trial. A week before surgery, the patient presented blood levels cholesterol (166 mg/dL, reference values: 200-239 mg/dL), triglycerides (80 mg/dL, reference values: 150-200 mg/dL), HDL 45 mg/dL (reference: >40 mg/dL) e LDL 104 mg/dL (reference: 130-159 mg/dL).

Hypnosis was obtained with target-controlled propofol administration (TCA) by intravenous infusion using a specific pump (Diprifusor, Astra-Zeneca, Detroit, USA), with initial infusion time of 30 seconds and adopting 2 mg/L as target concentration through out the surgery. For the therapeutic drug monitoring, a serial of 15 arterial blood samples were (2 mL/each) collected into tubes containing sodium EDTA as an anticoagulant at the following time points: 0 (immediately before dose administration), and 0.08, 0.25, 0.5, 0.75, 1, 2, 6, 7, 8, 10, 12, 14, 16 and 18 hours after the starting of infusion.

**RESULTS**

**Validation of the analytical method**

The analytical method described in the present study was validated based on confidence limit parameters for the measurement of propofol in plasma (Table 1). The specificity of the analytical method for propofol in biological matrices was evaluated by injection of the extracts from normal (n = 2), lipemic (n = 2), and hemolised (n = 2) plasma blanks into the chromatographic system. Good specificity was demonstrated, since endogenous compounds eluted from the chromatographic column did not interfere with the analysis under the conditions described above.

Blank plasma with internal standard solutions and spiked blank plasma were shown in chromatograms A and B, respectively, while patient plasma sample extract was illustrated in chromatogram C. Fig. 1 shows the chemical structures of propofol and thymol (internal standard) and also the chromatographic profile of purified plasma extracts containing the internal standard and propofol. Peaks were eluted at 12 min. (thymol) and 21 min. (propofol) guaranteeing the selectivity of the chromatographic analysis.

Peak areas were integrated for propofol and the internal standard (thymol) and the area ratio was applied for the construction of the daily cal-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>propofol</th>
<th>Unity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity, $r^2$</td>
<td>0.9977</td>
<td></td>
</tr>
<tr>
<td><strong>LOD, n=10</strong></td>
<td>0.05 mg/L</td>
<td></td>
</tr>
<tr>
<td><strong>LOQ, n=10</strong></td>
<td>0.10 mg/L</td>
<td></td>
</tr>
<tr>
<td>Absolute recovery (n=9)</td>
<td>93.9 %</td>
<td></td>
</tr>
<tr>
<td>Precision/within-day (n=18)</td>
<td>8.4 CV%</td>
<td></td>
</tr>
<tr>
<td>Precision/between-day (n=18)</td>
<td>8.8 CV%</td>
<td></td>
</tr>
<tr>
<td>Accuracy/within-day (n=18)</td>
<td>91.8 %</td>
<td></td>
</tr>
<tr>
<td>Accuracy/between-day (n=18)</td>
<td>93.0 %</td>
<td></td>
</tr>
<tr>
<td><strong>Short term stability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^a$ Extracts on the rack of autosampler (n=9)</td>
<td>7.1 %</td>
<td></td>
</tr>
<tr>
<td>$^b$ Standard solutions (n=9)</td>
<td>4.4 %</td>
<td></td>
</tr>
<tr>
<td>$^c$ Thawing cycles (n=27)</td>
<td>7.1 %</td>
<td></td>
</tr>
<tr>
<td>Long-term stability (n=108)</td>
<td>14.0 %</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Validation of analytical method for propofol measurements in plasma by HPLC-F. $r^2$: linear correlation coefficient; LOD: limit of detection; LOQ: limit of quantification.
The developed method showed high sensitivity (LOQ = 0.1 mg/L and LOD = 0.05 mg/L), linearity between 0.1 and 10.0 mg/L, a linear correlation coefficient of 0.9977, good recovery and acceptable accuracy and precision; in addition stability and robustness were guaranteed (Table 1).

The robustness of the method was determined based on the use of two different analytical C18 columns (5.6%), small variation in the pH ± 0.01 after little changes in the proportion of acetonitrile in the mobile phase (8.5%) and ± 0.1 mL/min changes on the flow rate (6.7%, Table 1). The authors investigated also the stability of propofol in plasma and in the organic extracts from the chromatography. Three consecutive freeze-thaw cycles showed good stability for propofol in the biological matrices stored in a freezer at -20°C. Analysis of the short-term stability of propofol (time and condition of analysis of extracts on the bench) demonstrated the absence of degradation of the drug in plasma extracts submitted to chromatographic analysis within a period of 24 h (vials on a tray); in addition the stability of standards of propofol and thymol in methanol after the storage at -20°C was guaranteed up to 12 months. Study of long-term stability showed the absence of degradation of propofol in biological matrices stored in a freezer at -20°C for 12 months. The variations obtained in the stability studies are showed in Table 1.

**Therapeutic drug monitoring**

Propofol plasma concentrations obtained during the infusion were: 2.2 mg/L (0.08 h), 2.6 mg/L (0.5 h), 2.4 mg/L (1 h), 3.3 mg/L (2 h), 3.2 mg/L (4 h). Maximum concentration of propofol in plasma was reached in 2 h (3.3 mg/L) and were maintained at 3.2 mg/L during the infusion in the perioperative period. Depth of anaesthesia expressed as bispectral index (BIS) was 36 (32-40) in that period up to the 4th h, and an adequate hypnosis was obtained. At the end of

**Figure 1.** Chemical Structure (propofol and thymol) on the top. Chromatographic profile of propofol in biological matrix extracts plus internal standard solution added (Thymol 25 ng/assay, 12 min) (A): Blank plasma; (B) Spiked blank plasma (Propofol 0.5 mg/L, 21 min); (C) Patient plasma sample.

**Figure 2.** Calibration daily curve accepted by the internal control to determine propofol (0.1-5.0 mg/L) in plasma samples. Average of two determinations

**Figure 3.** Case Report: Therapeutic Monitoring of Propofol Concentration and Effect assessments; Patient #FCS, tendency curves (—) Propofol plasma concentration versus time curve; (—O—) Drug Effect measured by BIS versus time. Average of three measurements.
surgical intervention, the infusion was interrupted and a declining curve was observed until the minimum propofol plasma concentration of 0.2 mg/L reached at the 18th h of the study. As illustrated in the Figure 3 an exponential decay was obtained after the interruption of drug infusion. In parallel, bispectral index (BIS) was monitored showing values as follows: 55 (6 h), 54 (7 h), 56 (8 h), 63 (10 h), 70 (12 h), 90 (15 h), 95 (20 h) indicating the return of BIS to the baseline with the patient awaked at the end of period of study.

DISCUSSION

Based on the specificity, selectivity and high sensitivity reached for the determination of propofol in whole blood and plasma, several analytical methods reported HPLC with fluorescence detection as a good choice, when compared with other procedures using HPLC-UV. Additionally, the electrochemical detection reported by Mazzi et al. and Dowrie et al., showed that its sensitivity is comparable to fluorescence detection for propofol determined in biological matrices.

Concerning the clean up procedures available to determine propofol in biological matrices, the precipitation of plasma proteins, solid phase or liquid-liquid extractions were reported previously.

The liquid-liquid extraction applied to whole blood, plasma or serum employing cyclohexane plus TMAH or TEAH or with pentane, proposed by Dowrie et al. were described in the literature. The stability of propofol in biological extracts revealed that those amines added to the solvent or even to the organic extract before the evaporation are required to increase drug recovery. When pentane was chosen, the recovery of drug extraction was decreased by comparison to cyclohexane.

In addition, procedures employing the precipitation of plasma proteins with acetonitrile or acetate) plus methanol or acetonitrile, added 2. It were also reported the use of other binary mixtures consisting of buffers (phosphate or acetate) plus methanol or acetonitrile, or even the ternary mobile phase proposed by El-Yazigi et al. In addition, another disadvantage of several methods described previously is related to different ranges the linearity obtained for the drug. Thus, different linearities make infeasible the monitoring of propofol in plasma of patients submitted to extensive surgeries, since higher propofol plasma concentrations (3-4 mg/L) were reached during the surgery by the target controlled infusion of the hypnotic drug, that declines up to very low concentrations at the end of the postoperative period. It must be mentioned also that the robustness and stability were investigated in the present study. The linearity, limit of quantification and limit of detection, precision and accuracy obtained in the present study are similar to those reported by Seno et al. Nevertheless, compared to our procedure, disadvantages are pointed in this method like the absence of an internal standard, the chromatographic analysis needs an oven with a premature elution of propofol.

The C18 column was employed, according to those reported in the previous studies, whereas the binary mobile phase consisting of organic solvent (methanol or acetonitrile) plus water. Even a oven was required for the heating of the column to keep at 37 °C or 40 °C, aiming to speed up the elution of the drug and its internal standard from the chromatographic column.

A binary mobile phase described in the present study consisted of acetonitrile plus water acidified with 0.05% glacial acetic acid, instead of 0.1% TFA, that permits to obtain a better selectivity by comparison to the method proposed by Plummer, since thymol and propofol were eluted respectively at 12 and 21 minutes, instead 2.8 and 4.5 minutes as a consequence of TFA added 2. It were also reported the use of other binary mixtures consisting of buffers (phosphate or acetate) plus methanol or acetonitrile, or even the ternary mobile phase proposed by El-Yazigi et al.

In addition, another disadvantage of several methods described previously is related to different ranges the linearity obtained for the drug. Thus, different linearities make infeasible the monitoring of propofol in plasma of patients submitted to extensive surgeries, since higher propofol plasma concentrations (3-4 mg/L) were reached during the surgery by the target controlled infusion of the hypnotic drug, that declines up to very low concentrations at the end of the postoperative period. It must be mentioned also that the robustness and stability were investigated in the present study. The linearity, limit of quantification and limit of detection, precision and accuracy obtained in the present study are similar to those reported by Seno et al. Nevertheless, compared to our procedure, disadvantages are pointed in this method like the absence of an internal standard, the chromatographic analysis needs an oven with a premature elution of propofol.

The linearity of 0.1 to 10mg/L obtained were adequate for drug monitoring in plasma, during the target controlled infusion since some patients can reach 3-4 mg/L in the extensive higher surgeries as the coronary artery bypass grafting with cardiopulmonary bypass or Offpump. Also the method showed sensitivity equivalent to 0.1 mg/L, good enough for the investigation of the washout of the hypnotic drug in the postoperative periods immediately and late surgery.
A report case of a patient submitted to heart surgery of revascularization (CABG-CPB) receiving propofol (TCI 3-4 mg/L) was investigated during the surgery and also after the interruption of infusion in the postoperative period. Bispectral index (BIS) monitored in this patient showed that adequate hypnosis was reached (BIS: 40) during the surgery guaranteed by the target drug infusion. After the interruption of infusion, plasma levels of propofol declined up to 0.2 mg/L at 20th h with the return of BIS to the baseline with the patient awaked at the end of period of study. The present method, besides being adequate for monitoring of plasma concentration of propofol of an adult patient submitted to heart surgery and can also be employed in pharmacokinetics studies and PK-PD modelling, also for high dose therapy.

Acknowledgements: The project was supported by the Brazilian foundation FAPESP and CNPq.

REFERENCES