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Article

Determination of Propofol Using High Performance Liquid Chromatography in Whole Blood with Fluorescence Detection

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High-performance liquid chromatography method for the determination of propofol has been developed and validated. Following a liquid extraction using ethyl acetate and hexane, samples were separated by reverse-phase high-performance liquid chromatography on an XBridge C_{18} column and quantified using fluorescence detection at an excitation of 276 nm and an emission of 310 nm. The mobile phase was a mixture of water (pH 4.0) and acetonitrile, with a flow rate of 1.5 mL/min. The standard curve ranged from 5–2000 ng/mL. Intra- and inter-assay variability for propofol was less than 10%, and the average recovery was greater than 95%. This assay is suitable for use in pharmacokinetic studies.

Introduction

Propofol (2, 6-diisopropylphenol) is a commonly used, rapidly acting injectable anesthetic with a very short duration of action, used for the induction and maintenance of anesthesia (1, 2). It can also be useful for sedation in patients as an adjunct regional anesthesia and for sedation in critically ill patients requiring mechanical ventilation (3). After receiving a single intravenous dose of propofol, the patient loses consciousness within 30–50 s and remains unconscious for about 4–6 min (3). It is rapidly gaining widespread utility for I.V. anesthesia and sedation in dogs and humans as it has a major advantage over other injectable anesthetic agents, that being the rapid and complete recovery that occurs even after relatively prolonged I.V. infusions (4). This property results from the rapid and extensive biotransformation of the parent compound to multiple inactive metabolites. This biotransformation occurs primarily in the liver with the metabolites being excreted in the urine (4), but elimination exceeds hepatic perfusion, suggesting extensive extra-hepatic metabolic elimination. Propofol's metabolites, which can be detected in plasma by high-performance liquid chromatography (HPLC), do not contribute to its sedating effects (5, 6). Studies indicate that propofol binds to whole blood, resulting in higher recorded propofol levels than in plasma alone (5–8). In order to achieve accurate levels, measurements in this biological sample are advisable (5–8).

Propofol levels have been reported using HPLC methods incorporating UV (6, 9–13), fluorescence (5–8, 13–20), mass spectrometry (3, 6, 13, 21–24) and electro-chemical detection (6). The use of mass spectrometer can produce results that are more sensitive and possibly simplify preparation however they are costly and may not be available in all laboratories. Methods have included a variety of extraction techniques. The use of solid-phase extraction cartridges result in higher extraction costs per sample (6, 12, 23, 24). The use of harsh organic solvents like cyclohexane and hexane (9–11, 14, 16, 19–22) and aqueous solutions like tetra methyl ammonium hydroxide and hydrochloric acid (9–11, 14-16, 19–22) can be avoided. The aim of this paper was to describe a sensitive and accurate method for extracting propofol from canine whole blood samples using HPLC.

Methods

Reagents

Propofol was purchased from U.S. Pharmacopeia (Rockford, MD), while 2,4-ditert-butylphenol was purchased from Sigma-Aldrich (Saint Louis, MO). All other reagent-grade chemicals were purchased from Fisher Scientific (Pittsburg, PA). Water was obtained from a Barnstead (Dubuque, IA) Nanopure Infinity ultrapure water system.

Chromatography

The chromatography system consisted of a 2695 separation module and a 2475 fluorescence detector (Waters, Milford, MA). Separation was achieved on a Waters XBridge C_{18} (4.6 x 250 mm, 5 μm) column preceded by a 5 μm XBridge C_{18} guard column. The mobile phase was an isocratic mixture of A: water (pH 4.0 with glacial acetic acid) and B: acetonitrile (31:69). All solutions were filtered through a 0.22-μm filter and degassed before their use. The water was replaced on a daily basis. The flow rate was 1.5 mL/min, and the column temperature was ambient. The fluorescence detector was set at an excitation of 276 nm and an emission of 310 nm with the gain at 10x.

Preparation of calibration standards

Propofol and 2,4-ditert-butylphenol (internal standard) were dissolved in methanol to produce stock concentrations of 100 μg/mL. Dilutions of the propofol stock solution were prepared to produce 1 and 10 μg/mL working stock solutions. Standards were aliquoted into 2-mL vials to prevent evaporation and cross contamination. All solutions were protected from light in bottles wrapped in aluminum foil and stored at −20°C. Standards were stable for 4 months at this temperature.

For preparation of calibration standards and quality control samples, appropriate volumes of stock solutions were evaporated and untreated whole blood added. The final concentrations for our standard curve were 5, 10, 25, 50, 100, 250, 500, 1000, 1500, 2000 ng/mL, and 7.5, 350, 800, and 1700 ng/mL for calibration standards. Calibration standards and control samples were treated the same as test samples. Linearity was

assessed by linear regression analysis. The calibration curve had to have a correlation coefficient of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except lower limit of quantification (LLOQ), which was set at 20%.

Sample extraction

Propofol was extracted from canine whole blood using a liquid extraction. Previously frozen blood samples were thawed, and 400 μL was placed into a 16 x 100 mm glass tube. Ten microliters of 2,4-ditert-butylphenol (internal standard, 100 μg/mL) was added followed by 1 mL of acetonitrile–methanol (75:25). The tubes were vortex mixed at a low speed for 10 s to mix solutions. They were covered with parafilm and placed in the refrigerator for 10 min. The tubes were then vortex mixed for 10 s and centrifuged at 2000 x g for 15 min. The supernatant was removed to a clean glass test tube.

In order to improve drug recovery, a second extraction was performed on the blood pellet by adding an additional 0.5 mL of acetonitrile–methanol. Samples were vortex mixed gently for 10 s and then centrifuged for 15 min at 2000 x g, and the supernatant was combined. The tubes were then centrifuged for 5 min to settle any particles that were suspended in the solution. The supernatant was placed in chromatographic vials and 40 μL injected into the HPLC.

Results and Discussion

Optimization of extraction procedure

Several extraction methods were tested to determine which procedure would provide the greatest recovery and best peak resolution. Methanol, cyclohexane, cyclohexane with tetra methyl ammonium hydroxide or tetra butyl ammonium hydroxide, hexane, and acetonitrile were tested and found to produce a lower recovery, decreased peak resolution, or both. A single extraction step using acetonitrile–methanol (ACN–MeOH, 75:25) was initially performed with poor recovery results. The protocol was changed to provide additional time for the extracting solution to lyse the blood cells, after which a second extraction was performed. The addition of a second extraction (0.5 mL ACN–MeOH) resulted in a 20% increase in propofol recovery.

Initial extractions used thymol as an internal standard (IS), but due to interferences from endogenous peaks in our control blood samples, it was discontinued. 2,4-Ditert-butylphenol was determined to be a suitable IS for the assay and the conditions used.

Chromatography was also improved by adjusting the type of buffer. Potassium dihydrogen phosphate and sodium phosphate buffers were used initially. Both buffers produced constituents that interfered with the elution of propofol and poor peak resolution. Water (pH 4.0) resulted in the best chromatographic resolution for all compounds used.

Results

Untreated blood samples for specificity testing were prepared in the same way as the study samples. Seven different untreated plasma samples were used in the pre-validation process, and a blank sample from each canine was included in the analysis. Endogenous components from plasma did not interfere with the elution of the compounds of interest. Figure 1 shows chromatograms of a blank canine blood sample (Figure 1A), 50 ng/mL blood standard (Figure 1B), and a canine blood sample after a dose of 2.5 mg/kg (Figure 1C). Retention times were 5.7 min for propofol and 9.1 min for 2,4-ditert-butylphenol.

The method for whole blood analysis produced a linear curve, for the concentration ranged used (5–2000 ng/mL) with a correlation coefficient of 0.9994. The mean slope, intercept and r^2 values are reported in Table I. Intra- and inter-assay relative standard deviation (RSD) for whole blood spiked with specific concentrations of propofol to determine accuracy and precision ranged from 2.3% to 8.2% (Table II).

Recovery was calculated by comparing the areas of propofol and the IS with those of directly injected analytes at concentrations of 5, 10, 25, 50, 100, 250, 500, 1000, 1500 and 2000 ng/mL. The average recovery was 95% for propofol and the IS (2,4-ditert-butylphenol). The lower limit of detection (LLOD) was 2.5 ng/mL, while the lower limit of quantification (LLOQ) was 5 ng/mL. Testing of the short term stability of the standards at concentrations of 10, 100, and 1000 ng/mL indicated there was less than 2% of drug loss after 24 h in the auto sampler or after 24 h in the refrigerator at 4°C.

Discussion

This HPLC method quantifies propofol from whole blood by combining a liquid extraction with fluorescence detection. Some methods involved in propofol analysis use mass spectrometry; however, mass spectrometry equipment is expensive and may not be readily accessible to most laboratories (3, 13, 21–24). This method produced a LLOQ that is equal to or in most cases is better than methods using UV detection (9–13), fluorescence detection (5, 7, 8), and some mass spectrometer methods (13, 22–25). The Plummer method (20) had an LLOQ of 2 ng/mL, which is lower than the Nolan (21) method and ours, which was 5 ng/mL. Nolan and Plummer's et al. methods (20, 21) required 1 mL of whole blood to achieve this level, while our method uses 400 μL of blood. The results were much higher than expected in some samples, and sample volumes as low as 100 μL were successfully used for detecting propofol levels. If a lower LLOQ is needed, the sample volume could be increased; however, it is more than adequate for our study purposes.

The extraction chosen is a straightforward and easily repeatable procedure. This method does not require as large a volume of organic solvents, such as cyclohexane, hexane, ethyl acetate, or harsh aqueous solutions of tetramethylammonium hydroxide (9–11, 14, 15, 20, 21) and hydrochloric acid (15). This method also eliminates the need for costly solid-phase extraction cartridges that Teshima (11) and Cohen (23) used. A recovery of 95% for both propofol and the IS is similar or better than other methods (3, 5, 9, 12, 16, 23).

The use of 2,4-ditert-butylphenol as an internal standard corrects for the intra- and inter- assay variability in the extraction. Stability studies indicate that samples are stable for 24 h after extraction at room temperature. In the event of an equipment

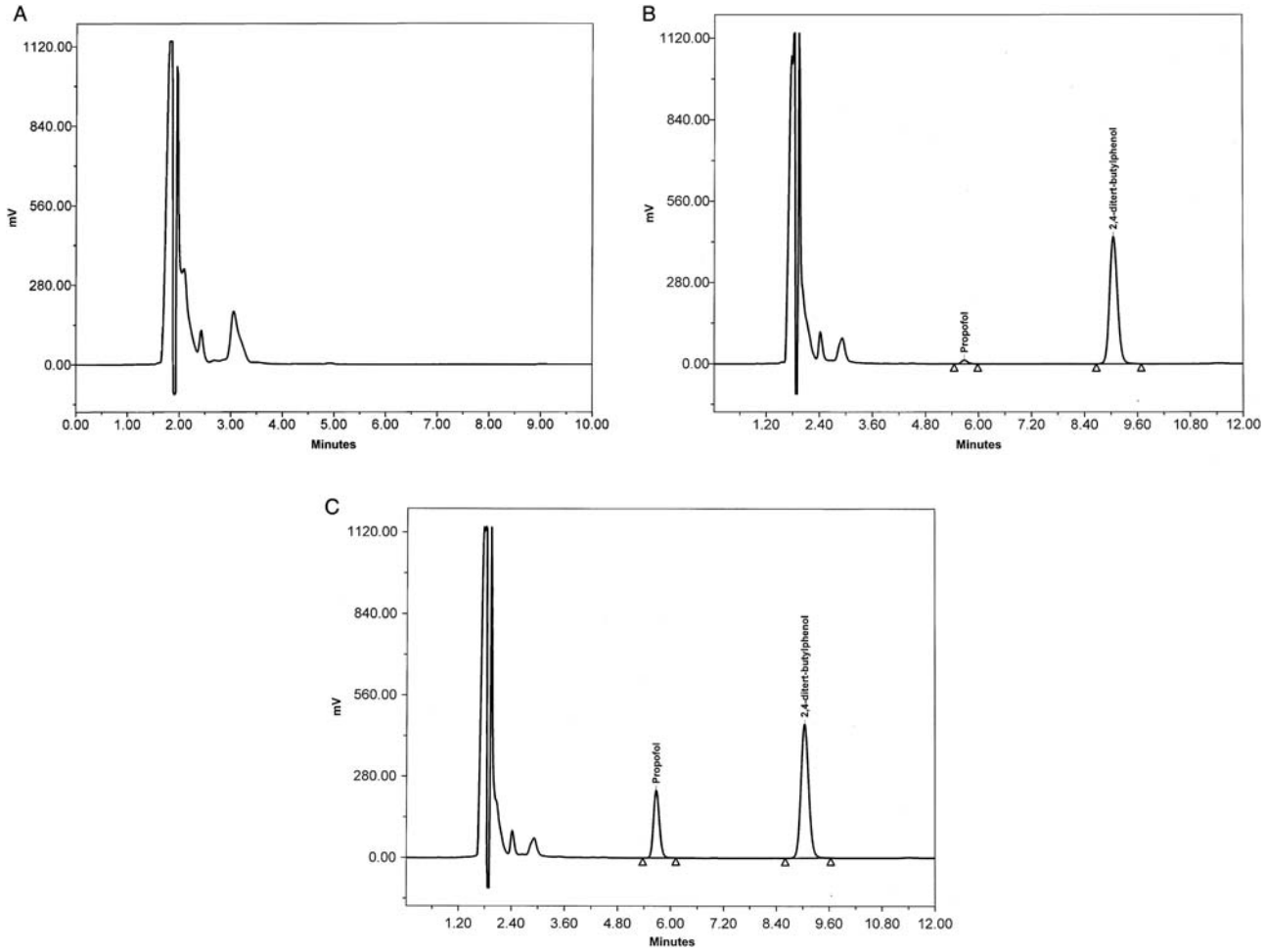

Figure 1. Chromatograms are representative of (A) blank canine blood, (B) canine blood spiked with 50 ng/mL of propofol and I.S. (2,4-ditert-butylphenol), (C) canine blood sample after an I.V. dose of 2.5 mg/kg of propofol. The propofol level, 30 min post injection, was 641.9 ng/mL.

Table I
Propofol Assay Linearity ($n = 4$)*

	Mean ± SD	RSD (%)
Slope	0.00039 ± 0.00003	6.48
Y-intercept	−0.00056 ± 0.00005	9.67
r^2	0.9994 ± 0.00051	0.05

* SD = standard deviation; n = number of curves; RSD = relative standard deviation; r^2 = correlation coefficient.

malfunction, samples could be reanalyzed. Freeze-and-thaw studies were not conducted; however several published studies have shown that up to three freeze-and-thaw cycles had no effect on propofol stability (8, 26, 27). This number of freeze-and-thaw cycles was not exceeded in any samples in our study. The method does have the potential to use only 100 μL for the sample size, making it potentially useful for small dog breeds and small exotic animals. The extraction chosen is a straightforward and easily repeatable procedure. The technologist can easily analyze 75 samples in one day. It is a rugged procedure with the column still in use after 1000 injections.

Table II
Propofol Intra- and Inter-Assay Linearity ($n = 4$)

Propofol intra-assay linearity (n = 4)

Concentration (ng/mL)	Concentration measured* (ng/mL)	RSD (%)
7.5	7.65 ± 0.26	3.40
350	356.8 ± 29.31	8.21
800	782.8 ± 39.05	4.99
1700	1729 ± 39.2	2.27
Propofol inter-assay linearity (n = 4)		
Concentration (ng/mL)	Concentration measured* (ng/mL)	RSD (%)
7.5	7.65 ± 0.52	6.74
350	353.7 ± 22.05	6.23
800	798.0 ± 29.26	3.67
1700	1717 ± 60.6	3.53

* Concentrations are mean ± standard deviation; n: number of samples; RSD: relative standard deviation

Conclusion

In conclusion, this analytical procedure was validated in terms of recovery, linearity, LLOQ, precision, and accuracy. The limit of quantification and recovery are more than adequate for use in pharmacokinetic studies. Our results indicate that this HPLC

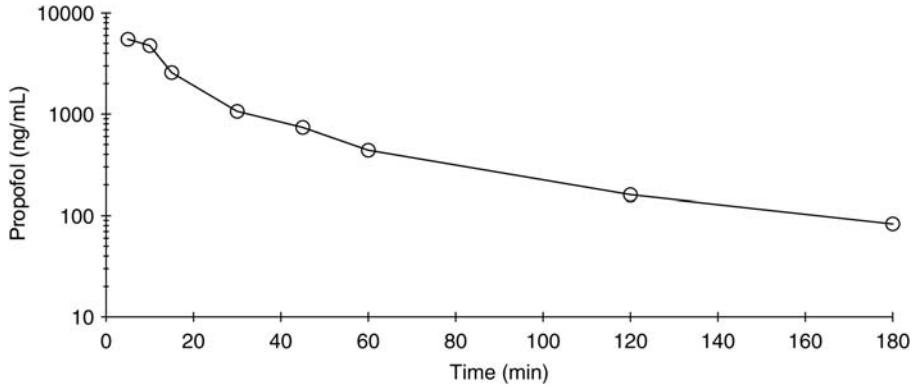

Figure 2. Blood concentration-time profile for propofol following I.V. administration of 2.5 mg/kg.

procedure is a reproducible method that provides consistent quantification of propofol in whole blood samples. This method has been used successfully in canine samples at this institution (Figure 2).

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