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Journal of Chromatography B, 678 (1996) 279–288

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic assay of propofol in human and rat plasma and fourteen rat tissues using electrochemical detection

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Received 1 May 1995; revised 27 September 1995; accepted 26 October 1995

Abstract

This paper describes a sensitive HPLC–electrochemical detection analytical method for determining the concentration of the intravenous anesthetic, propofol, in human or rat plasma or serum and a variety of rat tissues. Internal standard and drug are extracted from serum or plasma and other tissues with pentane. 2,6-*tert.*-Butylmethylphenol is used as internal standard. It includes a novel steam distillation procedure for separating the highly lipophilic propofol from skin and fat. The plasma/serum assay has a precision of 1–4% (C.V.) in the range 10 ng/ml to 1 μg/ml and permits the assay of 5 ng/ml from 0.1 ml of plasma/serum. The tissue procedure allows the estimation of 50 ng/g in 0.1 g of tissue for most of the major organs with less than 2% (C.V.) precision. This assay was used to measure propofol concentrations in plasma/serum and tissue samples in support of a project to develop a physiological pharmacokinetic model for propofol in the rat.

Keywords: Propofol

1. Introduction

Propofol, 2,6-diisopropyl phenol, is widely used to induce anesthesia in human and animal subjects. The purpose of the present investigation was to develop a sensitive assay for propofol in small serum or plasma samples that could be used for pharmacokinetic/pharmacodynamic studies in the rat, where multiple samples per animal are required. Sensitivity is particularly critical in the assay of rat plasma for pharmacokinetic/pharmacodynamic studies; sample size must be limited to 250 μl of blood (100–125 μl of plasma) to permit serial sampling while avoiding significant perturbation of the animal's blood volume, plasma proteins and hemodynamics. A second goal of this study was to develop an assay applicable to various rat tissues that could be used for physiological pharmacokinetic modeling.

A variety of methods have been published describing the assay of propofol in blood, plasma or serum. These procedures have generally employed HPLC with either UV or fluorimetric detection techniques both with [1] and without [2,3] precolumn derivatization; the most sensitive of these [2] combined liquid–liquid extraction with HPLC–fluorescence and was able to quantify 2 ng/ml from 1 ml of

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0378-4347/96/$15.00
SSDI 0378-4347(95)00475-0

plasma. One of these methods [3] included the assay of selected tissues. Two authors used HPLC coupled to an electrochemical detector, one using solid-phase extraction [4], another liquid–liquid extraction [5]. Both publications cite the use of larger than acceptable plasma sample volumes (0.5 to 1.0 ml) for small animal work; the more sensitive of the two had quantitation limits of 20 ng/ml [5]. Four investigators have reported using direct injections of plasma onto an HPLC, two injecting deproteinized plasma [6,7], another filtered plasma [8] and a fourth plasma without sample preparation [9]. Three of these were working at relatively high concentrations (0.1–20 μg/ml), with the objective of using their methods for clinical monitoring; the fourth [9] claims 5 ng/ml quantitation limits from 1.0 ml of sample. A more recent paper outlines the assay of propofol using gas chromatography–mass spectrometry of derivatized drug with quantitation limits of 0.2 ng/ml from 1 ml of plasma [10].

The assay described in this paper utilizes liquid–liquid extraction followed by an HPLC separation with electrochemical detection, exploiting the electrochemical detector's inherent sensitivity toward phenols. It permits the estimation of propofol concentrations of 5 ng/ml in 100 μl samples of human or rat serum or plasma (0.5 ng extracted) and 50 ng/g in 0.1 g rat tissue, where concentrations generally tend to be at least 10 to 100 times higher than plasma for a given dose.

2. Experimental

2.1. Materials

HPLC-grade pentane, methanol and acetonitrile used in the chromatography and extractions were purchased from J.T. Baker (Phillipsburg, NJ, USA). Phosphoric acid (85%), potassium phosphate monobasic, hydrochloric acid and 5 *M* sodium hydroxide used to prepare buffers were obtained from Fisher Scientific (Fairlawn, NJ, USA). Deoxycholic acid (sodium salt) used in the homogenization of fat and skin was obtained from Sigma (St. Louis, MO, USA). Isopropanol used in the evaporation of solvent was purchased from EM Science (Gibbstown, NJ, USA). 2,6-*tert.*-Butylmethylphenol (MTBP), the internal standard, was purchased from Aldrich (Milwaukee, WI, USA). Propofol was donated by Zeneca Pharmaceuticals (Wilmington, DE, USA).

2.2. Standard preparation

Standard stock solutions of propofol in 50% methanol were prepared in the range 10 ng/ml to 1 mg/ml; 50 to 200 μl of these solutions were added to blank serum, plasma or tissue homogenate (described below) to produce standards in the range of 0.5 ng to 100 μg. All standards were prepared such that they contained the same amount of methanol and tissue or plasma as the samples. Standards sets were ordinarily prepared over a twenty-fold range; five standards and one blank were prepared in each set.

2.3. Tissue preparation

The stomach, large and small intestine were rinsed with 0.05 *M* phosphate buffer (pH 7.0) upon collection. Just prior to homogenization, the remaining luminal contents were physically removed to the extent possible. All organs were collected on dry ice before transferring to a −17°C freezer. The liver was always harvested first, placed directly into a dry ice well and covered with dry ice. As much visible, adhering fat was removed as possible from organs prior to homogenization. Subcutaneous fat and hair were removed from the skin using a scalpel.

2.4. Tissue homogenization and sample extraction

Except for fat and skin (see special procedure below), 0.5 to 1.5 g frozen tissue (one whole organ if weight fell in this range), was thawed, subdivided into 2×2 mm pieces with a scalpel and added to a scintillation vial. Acetonitrile–0.05 *M* phosphate buffer (pH 7.0) (50:50, v/v) equal to four times the tissue weight was added and the tissue was homogenized to a uniform slurry (time dependent on tissue, three or four 10-s bursts of a Brinkman Polytron homogenizer, Model PT 10/35 with Model PTA 10TS generator). Scintillation vials containing tissue–buffer–acetonitrile were chilled to about 10°C prior to homogenization. Liver was subdivided while frozen and immediately homogenized to prevent enzymatic conversion of drug.

The following were added to a 15-ml glass culture tube with a PTFE-lined screw cap: plasma/serum (20 to 100 μl) or tissue homogenate (0.02 to 0.10 g tissue); an HPLC mixture of methanol–water (50:50, v/v) (to match total methanol in standards), 100 μl 0.5 *M* phosphate buffer (pH 7.0) (not added to plasma/serum); 100 or 200 μl internal standard in methanol–water (50:50, v/v); and water to bring the total volume to 1 ml. An aliquot of 4 ml pentane was added, the tube was capped and rocked on a Lab-Quake (Labindustries, Berkeley, CA, USA) at 40 cycles per min for 15 min. This was followed by centrifugation for 5 to 10 min at about 1500 *g*. The organic layer was transferred with a pasteur pipet to a second 15-ml culture tube; 1 ml of 0.1 *M* HCl was added to the pentane, the tube was capped and rocked for 10 min on a LabQuake and centrifuged for 5 min at 1500 *g*. The organic phase was transferred to an 8-ml glass screw cap culture tube with a pasteur pipet and 500 μl of a solution containing isopropanol–0.1 *M* NaOH (90:10, v/v) were added. The contents were evaporated to dryness (marked by the appearance of a white residue in the bottom of tube) at ambient temperature under a nitrogen gas stream, after rinsing the walls of the tube with about 1 ml of pentane when approximately 500 μl of liquid remained. The residue was reconstituted with 140 to 240 μl of mobile phase for serum/plasma and 490 μl for tissues. Aliquots of 10 μl of 0.6 *M* phosphoric acid were added to each reconstituted tube.

2.5. Preparation of fat and skin samples for chromatography

Fat was homogenized without subdividing; skin was subdivided in a manner similar to other tissues. Samples of fat or skin (0.1 g) were weighed directly into 100-ml round-bottom flasks and 20 ml of 5 m*M* sodium deoxycholate, 1 ml of 0.5 *M* phosphate buffer and 150 μl internal standard (10 μg/ml for skin and 100 μg/ml for fat) were added. The contents were homogenized with a Brinkman Model PT 10/35 homogenizer using two 4-s bursts for fat and about three 30-s bursts for skin, or until thoroughly shredded. Samples were prepared one at a time and steam-distilled immediately.

The contents of the flask were steam-distilled at a rate of 2 ml/min. Approximately 8 ml of distillate were collected in a 25-ml screw cap glass culture tube containing 8 ml of pentane. Four additional ml of pentane were then added to the tube and the tube was closed with a PTFE-faced cap. The condenser tip was rinsed with acetone–water (50:50, v/v) and distilled water. The still was cleaned between samples to prevent carryover of drug; three brief acetone washes followed by three brief water washes were drawn through the apparatus using a vacuum.

The tube containing distillate was rocked on a LabQuake for 15 min and centrifuged at 1500 *g* for 10 min. The organic layer was transferred to a 15-ml screw-cap glass tube with a pasteur pipet and 2 ml of 0.5 *M* NaOH were added. The tube was closed with a PTFE-lined cap, rocked on the LabQuake another 10 min and centrifuged at 1500 *g* for 5 min. The organic layer was again transferred with a pasteur pipet to another 15-ml tube and evaporated to about 4–5 ml at ambient temperature under a nitrogen stream. The contents of tubes containing extracted distillate from skin samples were transferred to an 8-ml screw cap tube; fat distillate/extract remained in the 15-ml tubes. A 500-μl volume of isopropanol–0.1 *M* NaOH (90:10, v/v) was added either to the 8-ml tube for skin or the 15-ml tube for fat. The contents of all tubes were evaporated to dryness under a nitrogen stream at ambient temperature, taking care to discontinue evaporation immediately after a white residue appeared in the bottom. Aliquots of 990 μl of mobile phase and 10 μl of 0.6 *M* phosphoric acid were added to the residue from skin samples and 10 ml of mobile phase and 10 μl of 0.6 *M* phosphoric to the residue from fat samples.

2.6. Chromatography

The HPLC system consisted of an ESA (Bedford, MA, USA) Model 420 dual tandem piston pump with a 0.01 to 2 ml/min pump head, an ESA pulse damper and an ESA Coulochem Model 5100A Electrochemical Detector with a Model 5010 dual electrode analytical cell and a Model 5020 guard cell, positioned between pump and injector. The guard cell was set at +0.85 V, detector 1 at +0.30 V and detector 2 at +0.80 V. The peak produced by oxidation at detector 2 was displayed on a Model 9176 dual-pen recorder (Varian, Walnut Creek, CA,

USA) and peak heights were hand-measured. Samples were hand-injected with a Rheodyne (Cotati, CA, USA) Model 7125 injector fit with either a 50- or 100-μl loop. Chromatography was carried out on a phenyl reversed-phase column (Rainin Microsorb MV, 100×4.6 mm I.D., 3 μm particle size, Rainin Instruments, Woburn, MA, USA). A temperature of 40°C was maintained with a Timberline Instruments column heater (Boulder, CO, USA).

Aliquots of 100 μl (in the range 0.5 ng to 10 ng) or 50 μl (for all other samples) were injected. The mobile phase consisted of methanol–50 m*M* phosphate buffer (pH 2.8) (60:40, v/v); flow-rate was 0.8 ml/min at 40°C. A constant light argon sparge was maintained with stirring during chromatography.

2.7. Assay recovery

Standards containing a combination of propofol and the internal standard (MTBP) at concentrations of 10 ng/ml, 25 ng/ml, 100 ng/ml and 1000 ng/ml were prepared in blank human serum. Similarly, homogenate containing 2.5 μg or 50 μg propofol and MTBP per gram of rat tissue was prepared for each tissue according to the procedure previously described. Ten 0.1-g samples of fat were spiked with 1 or 10 μg of propofol and MTBP while ten 0.1-g samples of skin were spiked with 0.5 μg of propofol and MTBP. Samples of fat and skin were spiked individually because of the difficulties encountered maintaining stable emulsions. Eight to ten 0.1-ml aliquots of serum standards or 0.1-g samples of tissue homogenate were evaluated for each tissue at each concentration. The tissue recoveries for both propofol and MTBP were calculated from the relationship between the peak heights of extracted standards and an appropriate reference injected directly.

2.8. Stability of drug in human serum and between-day variability

Aliquots (1 ml) of serum spiked at 50 ng/ml and of serum spiked at 1000 ng/ml were transferred to twenty one 1.5-ml borosilicate glass vials with a PTFE-lined cap and twenty one 1.5-ml polypropylene snap-top vials. Ten of these vials were used for the between-day variability and were assayed over the next two weeks; the remaining eleven from each group were assayed every two weeks for 22 weeks beginning on the fourth week. All vials were stored in a −17°C freezer. In order to assess the reproducibility of the assay in stored plasma containing propofol conjugates, the stability was similarly determined in plasma drawn at two different times from a patient receiving propofol.

2.9. Precision

The within-day variability of the serum assay was assessed in 100-μl aliquots of human serum spiked with 1, 2.5, 10 and 100 ng of propofol. Eight or nine replicates were assayed. Blank human serum samples spiked at 50 ng/ml and 1000 ng/ml, as described in Section 2.8, were assayed on ten separate days to determine day-to-day variability. The within-day variability of the tissue assay was assessed for selected tissues. Ten 0.1-g aliquots of brain and muscle homogenate (approximately 0.02 g tissue) containing 2.5 μg/g propofol, ten 0.1-g aliquots of brain and muscle homogenate (approximately 0.02 g tissue) containing 50 μg/g propofol, ten 0.1-g fat samples spiked with 1 μg of propofol and ten 0.1-g fat samples spiked with 10 μg of propofol were assayed according to their respective procedures.

3. Results

Fig. 1, panels 1 and 2 display the chromatograms resulting from HPLC analysis of 0.1 ml of human serum extracts, panel 1 the chromatogram of a blank serum extract and panel 2 the chromatogram of a 5-ng standard extraction. Fig. 1, panels 3 and 4 show the chromatograms resulting from HPLC analysis of the distillate/extract from 0.1 g of homogenized fat, panel 3 the chromatogram of a blank fat extraction and panel 4 the chromatogram of a 5-μg standard extraction. In both instances the internal standard and propofol elute close together, are well resolved and adequately separated from solvent and other peaks. The internal standard (MTBP) is retained on column about 7.5–8 min, propofol about 9–10 min.

The mean serum assay recoveries (% C.V.) were 68% (3.2), 69% (3.7), 72% (2.6) and 65% (2.6) for 10 ng/ml, 25 ng/ml,100 ng/ml and 1000 ng/ml

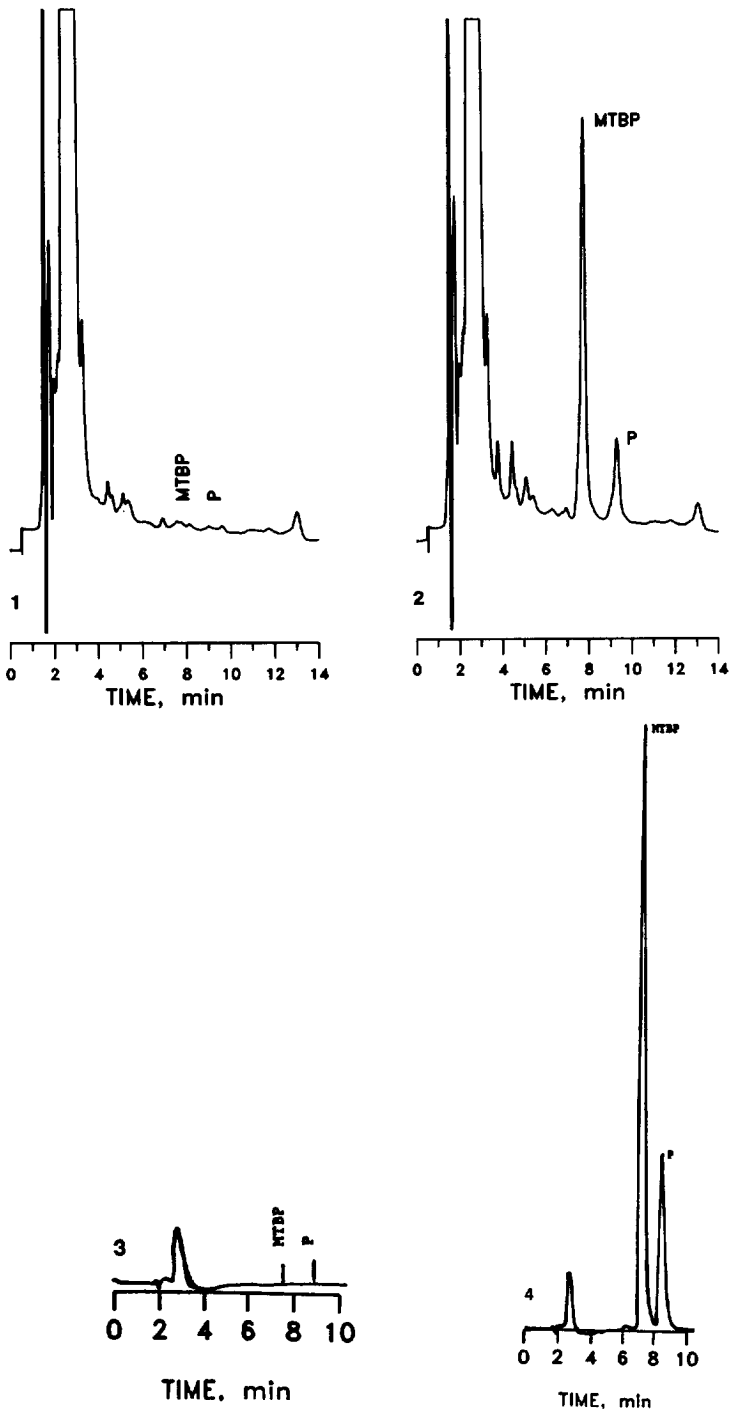

Fig. 1. Chromatograms for human serum extract and fat distillate/extracts. (1) Extract of 0.1 ml blank human serum. (2) Extract of calibration standard containing 0.1 ml of blank human serum, 5 ng of propofol (P) and 20 ng of internal standard (MTBP). (3) Distillate/extract of 0.1 g homogenized blank rat fat. (4) Distillate/extract of calibration standard containing 0.1 g homogenized blank rat fat, 5 μg of propofol and 15 μg of internal standard (MTBP).

propofol, respectively, and 67% (2.3), 67% (4.2), 72% (2.7) and 64% (2.8) for 10 ng/ml, 25 ng/ml, 100 ng/ml and 1000 ng/ml MTBP, respectively. Recoveries of propofol and MTBP were similar within each concentration range with neither MTBP nor propofol recoveries exhibiting any particular concentration dependence. Recoveries for both propofol and MTBP were likewise determined for fourteen rat tissues, including brain, heart, kidney, large intestine, liver, lung, muscle, pancreas, small intestine, spleen, stomach, testes, fat and skin. The recoveries for tissues were generally higher than those for serum, probably reflecting differences in methodology between tissue and serum extraction rather than any clear, direct relationship between concentration and recovery. Mean recoveries ranged from 75–82% for 2.5 μg/ml propofol and MTBP (C.V. 1.0%–4.0% for n=8–10) and from 76–85% for 50 μg/g (C.V. 1.1–4.5% for n=8–10), with propofol and MTBP recoveries matching one another closely and propofol and MTBP recoveries slightly higher at the higher concentration.

From 0.1 g of fat, 74 and 76% of the 1-μg propofol and MTBP spikes (n=10, C.V. 1–2%), respectively, were recovered, while 81 and 80% of the 10-μg propofol and MTBP spikes (n=10, C.V. 3–4%) were recovered from 0.1 g of fat. The higher concentrations were chosen to reflect the generally higher concentrations of drug in fat. From 0.1 g skin, 75 and 74% of the 0.5-μg propofol and MTBP spikes (n=10, C.V. 3%), respectively, were recovered.

All standard curves over each twenty-fold range, were well described by and fit the equation

$$y = bx^a + c$$

where y represents the peak height ratio (drug to internal standard), x represents the amount of drug (R>0.999) and a, b and c are constants. Propofol curves in serum were generally linear (a=1) in the low and middle calibration ranges (0.5–10 ng and 5–100 ng), but became somewhat non-linear (a= 0.95–1.00) in the upper (0.05–1.00 μg) range. Fig. 2 shows a calibration curve at the lowest range assayed for serum.

Table 1 summarizes the precision of the serum/plasma assay, which ranges from 1 to 4% across a 100-fold range. Because of the consistency obtained in the recovery samples (1–5% variability in peak height for all tissues at both concentrations), determining within-day variability for each tissue was deemed unnecessary. Table 2 shows the within-day variability of the assay at two concentrations in three selected tissues (brain, fat and muscle). Fig. 3, panels 1 and 2, presents graphically stability data obtained

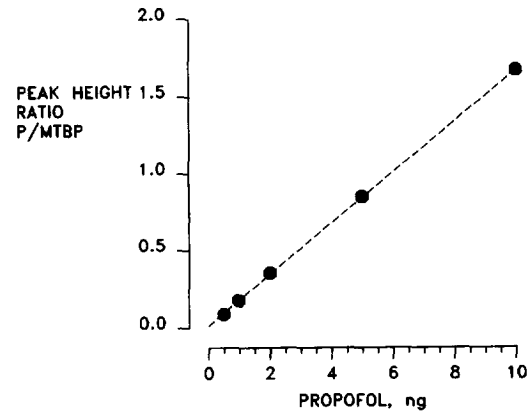

Fig. 2. Calibration curve of 0.5 ng to 10 ng propofol (P) and 5 ng internal standard (MTBP) extracted from 0.1 ml of human serum.

Table 1
Propofol plasma/serum assay precision

Amount of propofol (ng)	C.V. (%)
Within-day variability	
1	3.6
2.5	2.75
10	2.0
100	1.05
Between-day variability	
5	3.05
100	0.80

n=9 or 10. All extractions from spike of 100 μl of blank serum.

Table 2
Within-day variability of propofol assay for selected rat tissues

Concentration	C.V. (%)		
	Muscle	Brain	Fat
2.5 μg/g	1.1	2.2	–
50 μg/g	1.9	1.2	–
1 μg distilled from 0.1 g fat	–	–	1.6
10 μg distilled from 0.1 g fat	–	–	1.4

Extraction from approximately 0.02 g or 0.1 g of tissue; n=10.

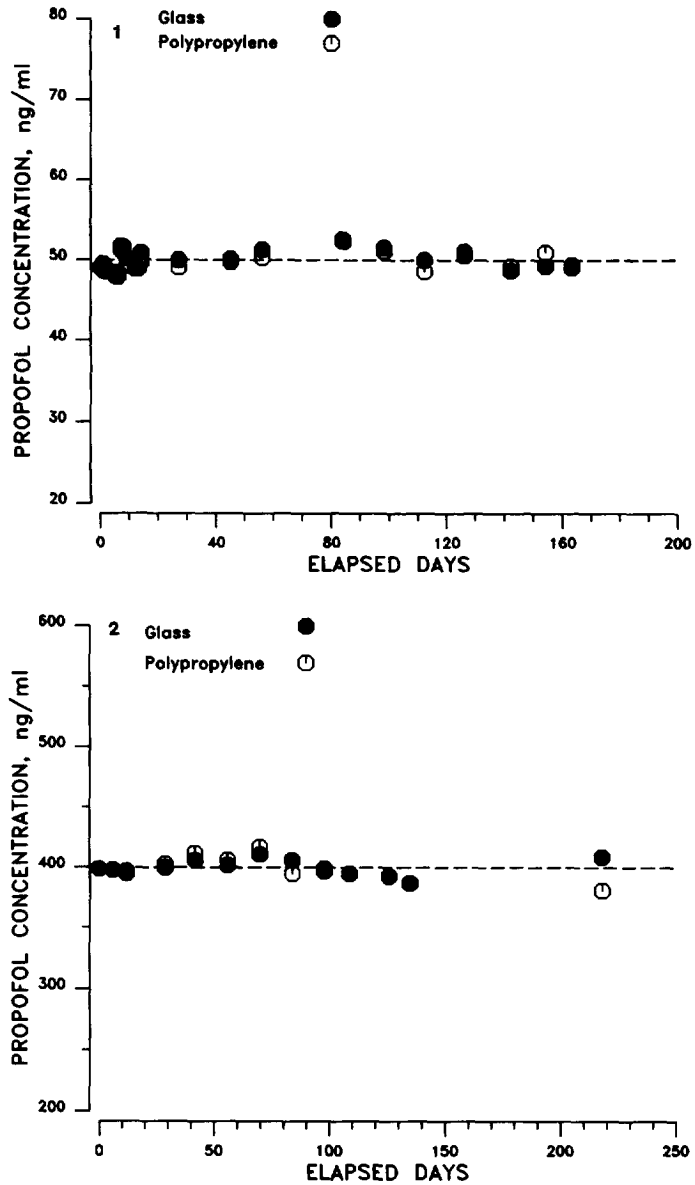

Fig. 3. Stability of propofol in frozen human serum. (1) Blank human serum spiked with 50 ng/ml propofol. (2) Serum drawn from a human surgical patient receiving propofol for anesthesia.

both on blank human serum spiked with propofol at 50 ng/ml and on human serum drawn from a surgical patient about 1.5 h after a propofol infusion of about 140 μg kg^{-1} min^{-1} was discontinued. The first graph includes data obtained from the first two weeks used to assess between-day variability. Propofol appears stable in frozen plasma stored either in borosilicate glass or polypropylene over 24 weeks, as the relative change in propofol concentration exhibits no particular trend. The stability was also carried out on a second spiked serum sample (1000 ng/ml) and serum containing a higher concentration of propofol drawn from a patient receiving propofol with similar results.

4. Discussion

In approaching the assay we present, we used Plummer's [2] extraction and isolation technique as a point of departure. To assure selectivity and minimize interference we continued with the use of a non-polar extraction solvent rather than resorting to a more universal solvent like ethyl acetate, including an acid wash step to eliminate endogenous amines. We were, however, interested in determining if electrochemical detection could be used in lieu of fluorescence to obtain sensitivity, given the relative ease with which phenols oxidize.

The hydrodynamic voltammogram (HDV) depicted in Fig. 4 demonstrates the feasibility of using electrochemical detection and shows the dependence of the curves on the composition of the mobile phase. Ideally the final potential would have been set at the voltage where the sigmoidal curve plateaus. Either using less methanol or setting a higher potential than we used (+0.8 V) would have enabled us to do this. However, using a higher voltage dramatically increased the background current and magnitude of the interferences. Searching for a column giving adequate separations and reasonable assay times using methanol–buffer (50:50) or lower proved fruitless. Switching to acetonitrile shifted the HDV further to the right and increased background current. Since propofol is virtually unionized up to pH 10, raising the pH from 2.8 to a higher value in the range 2–7, compatible with the column, would have had little influence on the oxidizability of propofol and could have had a large impact on the detector response toward certain ionizable interferences such as amines. We settled for a higher methanol content in the final composition and a phenyl column to obtain reasonable elution times while maintaining selectivity, working below the "top" of the HDV curve. This probably accounts for the slight non-linearity obtained at higher concentrations in the assay. A +0.3 V "screening" potential was also applied to the first electrode in the two electrode series to eliminate interferences arising from other drugs or endogenous substances such as catecholamines oxidizing at potentials below the voltage where the electrode begins to respond to propofol.

Plummer [2] used a quaternary ammonium salt to

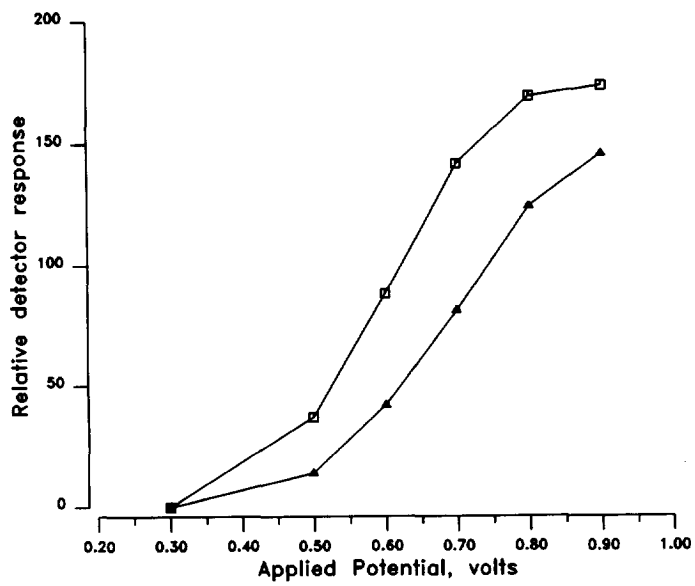

Fig. 4. Hydrodynamic voltammograms for propofol at two different mobile phase compositions. △=methanol–buffer (62:38), □=methanol–buffer (50:50).

make the organic phase basic and prevent loss of propofol and internal standard during the evaporation step. Quaternary ammonium salts injected on the HPLC–electrochemical detection system created enormous solvent peaks which seriously interfered with detection of internal standard and drug. Instead, we used dilute sodium hydroxide in isopropanol–water, which proved sufficiently miscible with the organic phase to serve the same purpose. Before injection on the chromatograph the sodium hydroxide was neutralized with phosphoric acid.

Most of the methods cited in the literature have used thymol for the internal standard; our assay uses MTBP, which, like propofol, is a hydrophobic, sterically hindered phenol, elutes closer to propofol and further from the solvent front than thymol, has an almost 1:1 molar response compared to propofol on the electrochemical detector and extracts similarly. The nearly identical recoveries of propofol and the internal standard at each concentration reflect this similarity. We believe these similarities have improved the reliability of the assay.

Owing to the lipophilicity of propofol, separating it from fat was highly problematic. Attempting to extract into an organic solvent always carried over unacceptable amounts of fat. Separation using solid-phase adsorbents also resulted in gross contamination with fat upon elution. Because of propofol's volatility on polar surfaces like glass, we reasoned that it might be readily separated from a dilute fat emulsion by steam distillation. Trapping and separation from water were achieved by placing pentane in the receiver tube. Maintaining an emulsion in the distillation flask was accomplished with deoxycholate. A 0.5 *M* NaOH wash step was included to minimize interference from more polar phenols (derived from inadvertent contact with plastics), which would also presumably distil. To facilitate cleaning, the steam distillation apparatus was designed so that acetone and water could be quickly drawn through it by vacuum.

At the lower concentrations very late peaks did present some interference to following chromatograms, usually about 1 to 1.5 h after injection. By carefully spacing chromatograms and injecting the minimum amount of sample necessary, most problems from delayed peaks were avoided. However, in the case of certain tissues, such as liver, the extent of the interference was such that samples had to be injected in batches of 4 to 6 samples followed by a waiting period for elution of late peaks.

The reproducibility and sensitivity of this serum/plasma assay compare favorably to the most sensitive of the HPLC methods and the inherent sensitivity of the electrochemical detector allows the use of very small samples (<0.1 ml). Interferences from matrix, not limitations imposed by detector sensitivity or noise have the greatest impact on the precision of this method at lower concentrations.

The tissue procedure described here permits the assay of propofol in all major organs to concentrations of 50 ng/g and provides a means for isolating the highly lipophilic propofol from skin and fat. Hundreds of rat tissue and plasma samples have been successfully assayed using this methodology to determine the tissue/plasma partition coefficients for a large array of rat tissues. The plasma to tissue partition coefficients obtained will be used in the development of a physiological pharmacokinetic model. Fig. 5 shows that a reasonable concentration–time profile is obtained for propofol in a rat after a short intravenous infusion of drug.

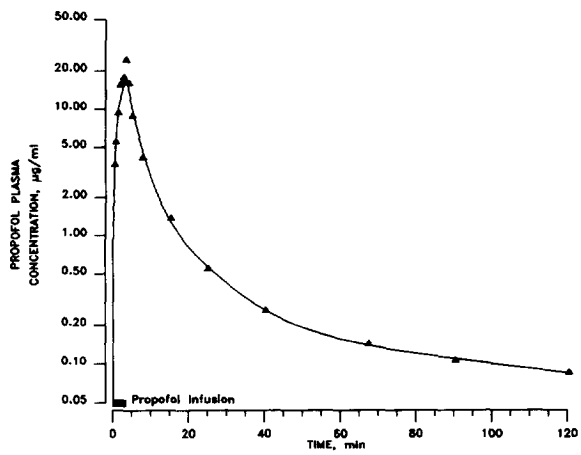

Fig. 5. Propofol plasma concentration versus time profile in a rat after a 20.76 mg/kg intravenous infusion of propofol given over 3.5 min.

Acknowledgments

This research was supported by a grant from Zeneca Pharmaceuticals. Zeneca kindly provided samples of propofol.

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