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Note

Direct plasma injection using internal surface reversed-phase high-performance liquid chromatography: feasibility study using propofol as a model compound

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Direct sample injection using internal surface reversed-phase (ISRP) high-performance liquid chromatography (HPLC) columns has recently been applied to the measurement of drugs in biological matrices [1–3]. The nature of the support permits lower-molecular-mass analytes to penetrate the pores where they are retained by a hydrophobic tripeptide bonded phase, while the plasma proteins are excluded and unretained by the hydrophilic exterior surface of the particles. Eliminating sample extraction steps simplifies both method development and routine sample analysis and, in addition, may yield improved precision, accuracy and recovery. Propofol was selected as a model compound for ISRP chromatography because of its innate fluorescence and UV absorption properties, the relatively high blood levels found during anesthesia and previous assay experience with the drug in this laboratory using a solvent extraction approach.

Propofol (the active agent of Diprivan® injectable emulsion) is a new intravenous anesthetic agent shown to be effective for the induction and maintenance of general anesthesia [4,5]. An induction bolus dose of 2.5 mg/kg followed by intermittent bolus injections of doses between 25 and 50% of the induction dose typically results in propofol concentrations ranging from 1 to 10 μg/ml during maintenance of anesthesia [6]. Methods currently employed to monitor maintenance pharmacokinetic studies involve protein precipitation and HPLC with UV [7] or fluorescence [8] detection.

This paper addresses the feasibility of direct-injection ISRP chromatography for the determination of propofol in human plasma at levels which have been shown to be hypnotic in man. The assay was validated for linearity, accuracy,

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precision and specificity. The procedure was also cross-validated against a previously reported HPLC assay employing fluorescence detection [8]. Future avenues for exploration of the propofol direct-injection ISRP chromatographic assay are discussed.

EXPERIMENTAL

Materials

Propofol (2,6-diisopropylphenol) was supplied by the ICI Pharmaceuticals Group (ICI Americas, Wilmington, DE, U.S.A.). Acetonitrile, methanol, tetrahydrofuran, 2-propanol and water were HPLC grade and were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Potassium phosphate, monobasic, was Baker analyzed grade (99.8%, J.T. Baker). Blood was obtained from apparently healthy human volunteers using Venoject® collection tubes containing potassium oxalate anticoagulant and sodium fluoride (Terumo, Elkton, MD, U.S.A.). The blood samples were centrifuged at 1000 *g* for 15 min and the plasma was separated and stored at −20°C.

Sample preparation procedure

An appropriate volume of propofol reference standard was diluted with methanol to produce a 1 mg/ml stock standard. This solution was further diluted with methanol to produce spiking standards of 500, 200 and 100 μg/ml. All spiking solutions were prepared fresh weekly. Plasma calibration standards were prepared fresh daily at 10, 5, 2 and 1 μg/ml by spiking 1 ml of blank plasma with 10 μl of the appropriate solution. A pooled plasma standard was prepared at 4 μg/ml to assess inter-day assay precision and accuracy. Aliquots from the pooled standard were stored at −20°C until they were assayed. Fresh whole blood (10 ml) was spiked at 3 and 9 μg/ml (30 and 90 μl of a 1 mg/ml spiking standard, respectively) to verify the accuracy of using plasma as the assay matrix. In accordance with the column manufacturer's recommendation, the samples were passed through 0.2-μm Nylon 66 filters (Micro Separations, Westborough, MA, U.S.A.) prior to HPLC injection to remove particulate matter.

Chromatographic instrumentation and conditions

The HPLC apparatus consisted of an Altex Model 110A pump (Beckman Instruments, Berkeley, CA, U.S.A.), a Varian Model 9090 autosampler (Walnut Creek, CA, U.S.A.), a Pinkerton® ISRP glycine–phenylalanine–phenylalanine stationary phase (GFF), 5 μm particle size, guard column and analytical column (10 mm×3.0 mm I.D. and 150 mm×4.6 mm I.D., respectively; Regis, Morton Grove, IL, U.S.A.) and a Spectroflow 783 UV detector equipped with a deuterium source to monitor absorbance at 270 nm (ABI Analytical, Kratos Division, Ramsey, NJ, U.S.A.). The mobile phase was 0.1 *M* potassium phosphate (pH 6.1)–acetonitrile–tetrahydrofuran (8:1:1). The mobile phase was filtered through a 0.45-μm Nylon 66 membrane filter and degassed by helium sparging before use. A mobile phase flow-rate of 1.0 ml/min was used in all experiments and all work

was performed at an ambient temperature of approximately 24°C. The sample injection volume was 10 μl.

Data acquisition and quantitation

Collection and analysis of the detector output (1 V/A.U.) was performed with a Microvax computer (Digital Equipment, Maynard, MA, U.S.A.) using VG Multichrom software (VG Laboratory Systems, Altrincham, U.K.). Assay values were calculated using the mean response factor (peak height of propofol/standard concentration) calculated across the range of the calibration curve.

Cross-validation method

The direct-injection ISRP technique was compared to an HPLC method which employed a cyclohexane extraction and fluorescence detection. The latter method is currently used in this laboratory for routine analysis of propofol in whole blood and has been described previously [8].

RESULTS AND DISCUSSION

High-performance liquid chromatography

The HPLC system was operated under isocratic conditions [mobile phase 0.1 *M* potassium phosphate (pH 6.1)–acetonitrile–tetrahydrofuran (8:1:1)] with detection at 270 nm. Previous reports have described HPLC methods for measuring underivatized propofol by UV detection (270 nm) [7] and fluorescence detection [8]. Because of the lower fluorescence quantum yield in the largely aqueous mobile phase, fluorescence detection gave no sensitivity advantage over UV absorbance.

The mobile phase conditions were adjusted to maximize selectivity and sensitivity in a manner similar to that described by Pinkerton et al. [2]. The organic modifier content (acetonitrile, tetrahydrofuran or 2-propanol), buffer pH and column length were optimized to resolve propofol from the solvent front and endogenous peaks in an acceptable run time. Chromatograms of a plasma blank, a 1 μg/ml spiked plasma standard and a 10 μg/ml spiked plasma standard are presented in Fig. 1.

The chromatographic specificity was tested by injecting blank plasma samples from twenty normal human subjects in duplicate. Plasma from subjects who had ingested acetaminophen, acetylsalicylic acid, ibuprofen, caffeine and nicotine approximately 1 h before blood sampling was also injected. No detectable propofol interferences were observed.

The ruggedness and reproducibility of the chromatographic system were also evaluated. In a typical run ($n=18$), an average propofol retention time of 17.6 min with a relative standard deviation (R.S.D.) of 0.9% was obtained. Injection of more than 120 plasma samples did not produce an irreversible increase in column back-pressure or a change in propofol retention time. However, it was necessary to back-flush the analytical column with water followed by acetonitrile after each daily injection sequence to restore the original back-pressure. In the course of a long chromatographic run (48 plasma injections), there was a revers-

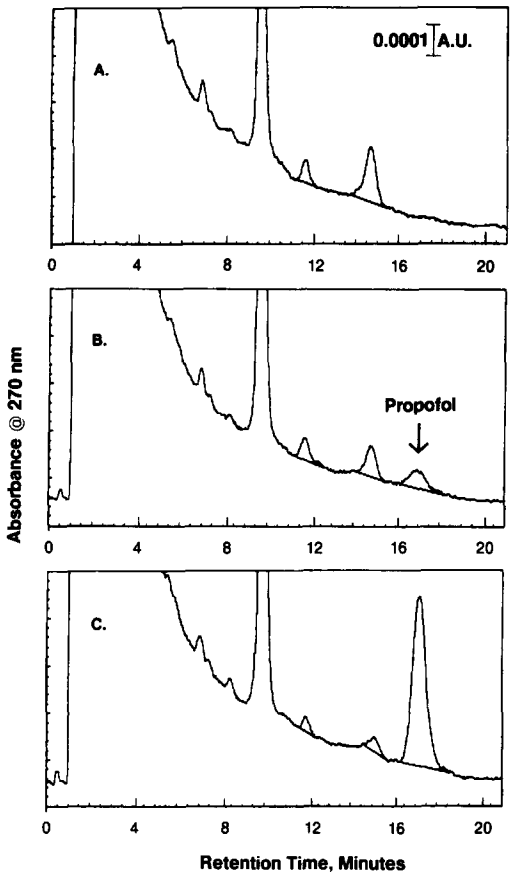

Fig. 1. Chromatograms of blank plasma and plasma spiked with propofol; conditions given in text. (A) Plasma blank; (B) plasma spiked with 1 μg/ml propofol; (C) plasma spiked with 10 μg/ml propofol.

ible increase in system back-pressure of 40 bar. It has been previously reported that such increases in back-pressure are primarily due to adsorption of high-molecular-mass plasma components on the stainless-steel column frits [9]. Inter-day differences in mobile phase composition produced changes in propofol retention times of up to 2 min. This did not significantly alter resolution or assay sensitivity. Sensitivity of ISRP column-based methods to inter-day differences in mobile phase composition has been noted by the column manufacturer [10].

Assay linearity

Spiked plasma calibration standards were analyzed in duplicate on each of four days at 1, 2, 5 and 10 μg/ml. Response factors (RF) were calculated as the ratio of the propofol peak height to the spiked plasma concentration. The results are presented in Table I. The average R.S.D. about the mean RF for the four days was 7.7%. As expected, precision increased as the standard concentration was increased. The signal-to-noise ratio for the 1 μg/ml standard was consistently ≥ 3. On three days, plasma standard peak heights were compared to analytical

TABLE I

SPIKED PLASMA CALIBRATION STANDARD PRECISION

RF is the response factor, as defined in text. Each value listed here represents the average of two injections of spiked plasma.

Spiking level (μg/ml)	Day 1		Day 2		Day 3		Day 4	
	RF	R.S.D. (%)	RF	R.S.D. (%)	RF	R.S.D. (%)	RF	R.S.D. (%)
1.00	27.7	0.9	32.4	17	36.6	9.0	26.6	3.2
2.00	29.6	6.7	29.7	3.6	36.1	3.2	27.5	1.9
5.00	32.9	1.5	34.0	0.4	35.0	4.8	28.8	3.9
10.0	33.2	0.4	36.2	1.5	35.6	1.5	31.7	4.2
Mean	30.9		33.1		35.8		28.7	
R.S.D. (%)	8.4		10		4.5		7.8	

TABLE II

POOLED PLASMA STANDARD PRECISION AND ACCURACY

Theoretical concentration = 4.00 μg/ml.

	ISRP direct-injection assay results (μg/ml)				Pooled estimate of precision and accuracy
	Day 1	Day 2	Day 3	Day 4	
Assay 1	3.92	4.22	3.85	4.13	
Assay 2	3.85	4.04	3.72	4.11	
Mean	3.89	4.13	3.79	4.12	3.98
R.S.D. (%)	1.2	3.1	2.4	0.3	4.3
Recovery (%)	97	103	95	103	100
	Cyclohexane extraction assay results (μg/ml)				Pooled estimate of precision and accuracy
	Day 1	Day 2	Day 3		
Assay 1	3.40	3.88	3.99		
Assay 2	4.50	4.28	4.09		
Assay 3	3.89	3.97	3.95		
Mean	3.93	4.04	4.01		3.99
R.S.D. (%)	14	5.2	1.7		7.6
Recovery (%)	98	101	100		100

standards prepared in mobile phase to determine absolute recovery. Pooled recoveries (R.S.D. in parentheses) for the 1, 2, 5 and 10 μg/ml standards were109% (15%), 97% (8.2%), 107% (7.5%) and 107% (6.7%), respectively.

Assay precision and accuracy

Inter-day precision and recovery were evaluated by analyzing aliquots from a pooled 4 μg/ml spiked plasma standard in duplicate on four days. The propofol

concentration for each analysis was calculated based on the mean RF for the calibration standard curve prepared on that day. The pooled results (Table II) demonstrated good precision (4.3% R.S.D.) and accuracy (100% relative recovery).

Cross-validation study

The pooled 4 μg/ml plasma standards were also analyzed by the previously reported HPLC method using cyclohexane extraction and fluorescence detection [8]. Triplicate assays were performed on each of three days and the results are presented in Table II. The pooled estimates of accuracy (100% relative recovery) and precision (7.6% R.S.D.) were comparable to the direct-injection ISRP method.

Plasma from whole blood spiked at 3 and 9 μg/ml was also assayed by both methods. The mean assay results by direct-injection ISRP chromatography (2.84 μg/ml, 1.7% R.S.D.; 8.06 μg/ml, 3.5% R.S.D.; $n=2$) were in good agreement with those obtained using the extraction-based method (2.97 μg/ml, 4.9% R.S.D.; 7.76 μg/ml, 6.3% R.S.D.; $n=3$). Plasma from the 9 μg/ml whole blood spike displayed a significant degree of hemolysis due to the volume of methanol added (90 μl). The lower recovery of this sample is in agreement with preliminary findings that hemolysis and clotting can alter the plasma/erythrocyte partition ratio of propofol to less than 1.

CONCLUSIONS

The applicability of a direct-injection HPLC method to measure propofol in plasma was demonstrated. The method is rugged and exhibits adequate precision and accuracy at hypnotic levels (1–10 μg/ml). Assay results in this range were in good agreement with a previously reported solvent extraction method.

An area for further investigation involves application of the assay to human clinical samples. The present procedure may be sensitive to changes in the binding of propofol to formed elements in the blood. Therefore, plasma propofol determinations on human clinical samples should be conducted using heparin as the ideal universal anticoagulant to maintain red blood cell integrity [11]. To provide further cross-validation and confirm that the plasma/erythrocyte ratio for propofol is 1, clinical samples drawn in heparinized tubes could be analyzed by both the cyclohexane extraction procedure and the direct-injection ISRP technique. For the latter, the plasma would be separated for analysis.

Methods employing solvent extraction report limits of detection of 2 ng/ml [8] and 100 ng/ml [7] versus 1 μg/ml with the current method. One promising approach to improve sensitivity would be to use an ISRP column as a concentrator pre-column in a manner previously described for determination of antidepressants in human serum [12,13]. Through column switching, a ten- to twenty-fold increase in maximum plasma injection volume was obtained by directing flow from the ISRP pre-column to waste initially to eliminate plasma proteins, then routing the retained analytes onto a conventional reversed-phase column using a stronger aqueous–organic mobile phase.

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