

Laboratory Report

Gas Chromatographic Assay for Free and Total Plasma Levels of Thiopental

Karl E. Becker, Jr., M.D.*

A rapid gas chromatographic assay for the determination of free and total plasma thiopental is described. Free thiopental was obtained by ultrafiltration through Amicon Centriflo membrane cones. Gas chromatographic assay utilized secobarbital as an internal standard and employed on-column methylation of the barbiturates to improve peak resolution. In 73 blood samples from 22 patients total thiopental concentrations ranged from 4.2 to 134 $\mu\text{g/ml}$ plasma, with a mean of 28 $\mu\text{g/ml}$. Free thiopental values ranged from 5.6 to 22.7 per cent of total, with a mean of 13.7 per cent free thiopental and a standard deviation of 3.2 per cent. At a total thiopental level of 10 $\mu\text{g/ml}$, unbound thiopental averaged 10.7 per cent with ultrafiltration, compared with 11.5 per cent with equilibrium dialysis. Assays of thiopental by gas chromatography and ^{14}C scintillation counting gave similar results. There were progressive increases in the percentages of thiopental that were unbound when thiopental was added to plasma, purified crystalline albumin (4.5 g/l), normal serum albumin (5 g/l), and a solution of purified protein fractions (5 g/l). Differences in protein binding determined by this method and previously reported methods are discussed. (Key words: Measurement techniques, gas chromatography; Anesthetics, intravenous, thiopental.)

IN THIS REPORT, a rapid method for determining the free and total concentrations of thiopental is described for the first time. Comparisons are made with the more lengthy method of equilibrium dialysis.

* Assistant Professor of Anesthesiology.

Received from the University Hospital, The University of Mississippi Medical Center, Jackson, Mississippi. Accepted for publication June 10, 1976. Supported in part by a grant from American Society of Anesthesiologists.

Address reprint requests to Dr. Becker: The University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216.

Methods and Materials

Plasma Preparation. Blood samples (10 ml) were collected anaerobically in heparinized syringes. Since the addition of exogenous buffers would dilute the sample and possibly interfere with protein binding, CO_2 gas (1 ml) was added to adjust the pH to 7.35–7.45 at room temperature. Syringes were centrifuged and plasma removed.

Ultrafiltration. Free plasma levels were determined by ultrafiltration (UF) through Amicon PM 50 cones (Amicon Corporation, Lexington, Massachusetts 02173). Plasma or plasma fractions (4.5–5 ml) were placed in the ultrafiltration cones. The centrifuge chamber was insufflated with 5 per cent CO_2 gas and approximately 1 ml of ultrafiltrate collected by centrifugation at 1,200 rpm for 15 minutes. Final pH was 7.4–7.5.

Extraction Procedure. To the 1-ml sample of plasma or protein-free eluant in a screw-capped culture tube were added either 20 or 2 μg , respectively, or an internal standard, sodium secobarbital, and 0.2 ml of 2 \times HCl (final pH < 2). Five ml of nanograde toluene were added and tubes extracted for 10 min on an aliquot mixer and centrifuged. A 4.5-ml amount of the toluene layer was transferred to a nipple tube (Concentratube, Laboratory Research Company, Los Angeles, California; 90036). The nipple tube was placed on a vortex mixer and 25 μl of 40 per cent trimethylphenylammonium hydroxide (TMAH) in methanol were slowly added. The tube was centrifuged, and then 1 μl of the TMAH solution injected into the gas chromatograph over a 10-sec interval. The TMAH provided on-column methylation of the barbiturates, which improved resolution and decreased analysis time.

Gas Chromatography. Gas chromatography (GC) was performed utilizing flame ionization detection and a 1.83 m \times 2 mm ID glass column containing 3 per cent SE-30 on 80/100 mesh Chromasorb W, HP, DMCS, AW. Injection port and detector temperatures were \pm 70 C, oven temperature, 140 C. H_2 , N_2 , and air flow rates were 40, 40, and 400 ml/min, respectively.

Determination of Thiopental Concentrations. High- and low-level standard curves were constructed using known amounts of crystalline acid thiopental ($2 \times$ acid-precipitated, ether-extracted) in 0.1 M $NaHCO_3$ buffer, pH 9, and 20 μ g and 2 μ g standards of sodium secobarbital. Extraction of thiopental from $NaHCO_3$ buffer and plasma was better than 98 per cent. Linear standard curves were determined by least-squares regression analysis of the ratio of peak heights of thiopental to secobarbital. Correlation coefficients were consistently greater than 0.998 and r^2 for linear curves greater than 0.995. Unknown thiopental concentrations were determined by comparing the ratio of peak heights of thiopental to secobarbital with the appropriate standard curve.

Comparison of Ultrafiltration with Equilibrium Dialysis. Known amounts of thiopental were added to undialyzed plasma or to plasma fractions dialyzed against Sorensen's phosphate buffer. Ultrafiltration was performed as detailed above. For equilibrium dialysis (ED), 6-ml amounts were placed in 0.64-cm diameter cellulose membrane tubing (Dialyzer Tubing, #8, Curtin Matheson Scientific, Incorporated) and then dialyzed against an equal volume of Sorensen's phosphate buffer, pH 7.4, for 24 hours, with continual mixing on a tilt-top mixer. Ultrafiltration was performed at room temperature. Equilibrium dialysis was performed at 4 C, room temperature, and 37 C. All experiments were performed in duplicate or triplicate.

Determination of Per Cent Free Thiopental. With ultrafiltration, the percentage of thiopental that was free (*i.e.*, unbound) was determined by dividing the concentration of thiopental in the ultrafiltrate by the total thiopental concentration multiplied by 100. With equilibrium dialysis, percentage free

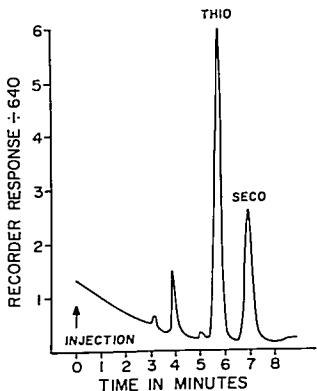


FIG. 1. Typical chromatogram of thiopental and secobarbital. The peaks represent the responses from plasma containing 40 μ g/ml thiopental and 20 μ g/ml secobarbital.

thiopental was determined by dividing the outside concentration of thiopental by the inside concentration of thiopental after dialysis.

Protein Leakage Through Ultrafiltration Cones and Dialysis Membranes. Protein leakage of plasma was determined spectrophotometrically by analysis of ultrafiltrates at 280 μ . There was 2 to 4 per cent protein leakage through the cones; protein leakage through dialysis tubing was 1 to 2 per cent. This material was precipitable with 10 per cent trichloroacetic acid.

Assays with Thiopental-2- ^{14}C . Thiopental-2- ^{14}C was obtained from ICN, Isotope Nuclear Division, Irving, California 92664. Radioactive purity was greater than 98 per cent. Two nanocuries of thiopental-2- ^{14}C (0.06 μ g per ml of plasma or plasma fractions) were used along with various amounts of cold thiopental. One-milliliter amounts were added to 10 ml of Aquasol (New England Nuclear, Boston, Massachusetts 02118) and scintillation counting performed. All samples were quench-corrected.

Determination of Thiopental by Spectrophotometry. In several experiments, thiopental was assayed at 305 μ after extract

TABLE 1. Comparison of Ultrafiltration and Equilibrium Dialysis; Comparison of Gas Chromatographic and Thiopental-¹⁴C Assays

Experiment	Assay Method	Thiopental Added ($\mu\text{g/ml}$)	Average Per Cent Free	
			UF	ED
1-5A	GC	10	10.7 $\pm 1.3^*$	11.5 $\pm 1.9^*$
5B	Thio- ¹⁴ C	10	12.2	14.9
6A	GC	25	13.2	15.7
6B	Thio- ¹⁴ C	25	13.3	15.8

* Mean \pm standard deviation of gas chromatographic assays in experiments 1-5A. No significant difference between the means.

tion into toluene (5 ml), with re-extraction into 0.1 N NaOH (2 ml).

Results

Gas Chromatographic Assay. A typical GC curve is shown in figure 1. The peaks were smooth and assay time was short. There was no interference from plasma proteins or drugs used as premedicants. Assay was sensitive to thiopental levels of less than 0.2 $\mu\text{g/ml}$. Thiopental concentrations determined by gas chromatography, ¹⁴C counting, and ultraviolet spectrophotometry were similar, but plasma and reagent blanks with the last method were high.

Per Cent Free Thiopental: Patient Studies. Seventy-three samples from 22 informed patients were analyzed. Total levels ranged from 4.2 to 134 $\mu\text{g/ml}$ plasma, with a mean of 28 $\mu\text{g/ml}$. Values for free thiopental ranged from 8.6 to 22.7 per cent of total, with a mean of 13.7 per cent free, standard deviation 3.2 per cent.

Ultrafiltration versus Equilibrium Dialysis. In table 1 are shown the results of equilibrium dialysis and ultrafiltration experiments on plasma assayed by gas chromatography and ¹⁴C counting. Percentages of free thiopental at a total level of 10 $\mu\text{g/ml}$ with the gas chromatographic assay averaged 10.7 per cent for ultrafiltration and 11.5 per cent for equilibrium dialysis. These differences were

not significant by t test. Results of gas chromatographic assays and ¹⁴C counting agreed to within 0.7 per cent. When the effects of temperature of the experiment were studied, a slightly greater percentage of free thiopental (1-2 per cent greater) was found at 37 C versus room temperature, and 2.5-5 per cent less free thiopental was found at 4 C. In table 2 is shown the binding of thiopental to a solution of purified protein fractions (5 g/l, Plasmanate, Cutter Laboratories) when the amount of thiopental was increased. By two-way analysis of variance, differences between results obtained with ultrafiltration and equilibrium dialysis were not significant ($P < 0.6$), but differences in percentages of free thiopental with different total thiopental concentrations were significant ($P < 0.001$). However, with least-squares regression analysis, there was poor correlation ($r = 0.66$) of percentage free versus total thiopental concentrations, with $r^2 = 0.43$ for linear curves and $r^2 = 0.49$ for quadratic curves.

Thiopental Binding to Plasma Fractions. Binding of thiopental to commercial plasma fractions and binding to purified human albumin were studied. Commercial fractions had to be well dialyzed against Sorensen's phosphate buffer prior to use, since there was considerable leakage (16 per cent) of protein (trichloroacetic acid-precipitable) through both dialysis membranes and ultrafiltration cones when dialysis was not performed prior to the experiment. The results are shown in

TABLE 2. Effect of Amount of Thiopental on Binding to Purified Protein Fractions*

Thiopental Added ($\mu\text{g/ml}$)	Per Cent Free Thiopental	
	Ultrafiltration	Equilibrium Dialysis
1	18.3	18.3
2.5	16.6	17.9
5	18.2	19.0
10	19.8	19.8
20	19.1	19.8
40	20.5	19.8
80	21.2	20.6

* Purified protein fractions. Plasmanate (Cutter Laboratories), 5 g/l, dialyzed overnight against Sorensen's phosphate buffer, pH 7.4; 2 nCi thiopental-¹⁴C (0.06 μg) added to each ml of protein.

table 3. There were progressive increases in percentages of free thiopental when thiopental binding was studied in plasma, 4.5 g/l crystalline human serum albumin (Sigma Chemical Corporation), 5 g/l normal serum albumin (Buminate 5%, Hyland, Costa Mesa, California 92626), and a solution of 5 g/l purified protein fractions (Plasmanate, Cutter Laboratories).

Discussion

Ultrafiltration with subsequent assay by gas chromatography is a rapid, accurate method of determining total and free plasma thiopental concentrations. Total analysis time for five plasma samples is less than six hours, and the ultrafiltration requires less than an hour, compared with the more lengthy method of equilibrium dialysis. This gas chromatographic assay, utilizing on-column methylation, is accurate, sensitive, and rapid, with no interference from plasma proteins, amino acids, or drugs routinely used as premedicants. Assay by gas chromatography gave results essentially identical to those of assay by ¹⁴C scintillation counting and by ultraviolet spectrophotometry. Assay at room temperature and assay at 37°C had only slightly different results. Plasma was found to bind thiopental better than purified albumin or albumin containing plasma fraction. This was expected, since drug binding is not solely due to the albumin fraction of plasma and since there is some denaturation and polymerization of albumin in commercial preparations.

Ultrafiltration results were not corrected for protein leakage or drug binding to the cones since: 1) no significant change in the percentages of free thiopental was found when corrections were made for protein leakage and for the 5–10 per cent binding of thiopental to membrane cones that occurred when thiopental was suspended in phosphate-buffered saline solution; 2) the effect of protein in decreasing binding of thiopental to membrane cones could not be determined from these experiments, thus making corrections tenuous. Finally, determinations of percentages of free thiopental by

TABLE 3. Thiopental Binding to Plasma Fractions

Plasma Fraction	Average Per Cent Free Thiopental	
	Ultrafiltration	Equilibrium Dialysis
Human serum albumin*	15.5	15.7
Human serum albumin†	16.7	20.1
Normal serum albumin‡	17.5	20.4
Purified protein fractions‡	19.8	19.8

* Human serum albumin, 4.5 g/l, purified and crystallized, Sigma Chemical Corporation.

† Normal serum albumin, 5 g/l, Buminate 5 per cent, Hyland Laboratories.

‡ Purified protein fractions, 5 g/l, Plasmanate, Cutter Laboratories.

All fractions dialyzed against Sorensen's phosphate buffer prior to use.

ultrafiltration and equilibrium dialysis agreed so well that corrections for protein leakage and membrane binding were not considered necessary.

More binding of thiopental was found than previously found by others. Brodie *et al.*² used equilibrium dialysis and a spectrophotometric assay to study thiopental binding to plasma. At concentrations of 10–50 μg/ml, 37°C, they found 75 per cent binding of thiopental. Ghoneim and Pandya,³ studying ten healthy patients and using equilibrium dialysis at 4°C and a spectrofluorometric assay, found 72 per cent binding of thiopental. Mark *et al.* found thiopental binding of 79–81 per cent using equilibrium dialysis and a spectrofluorometric assay with total thiopental concentrations of 10 μg/ml. However, in another experiment, Dayton and co-workers,⁵ using human plasma and equilibrium dialysis at 37°C, ¹⁴C scintillation counting for thiopental concentrations below 6 μg/ml, and spectrofluorometric assays for concentrations above 6 μg/ml, found thiopental binding to plasma of 85–90 per cent at concentrations below 6 μg/ml but 75 per cent binding above this level. Finally, in a recent review article on drug binding, Koch-Weser and Sellers,⁶ by extrapolating the data of Dayton *et al.*,⁵ found 87 per cent binding of thiopental to human plasma at 37°C. In my work, 86.3 per cent binding (13.7 per cent unbound) thiopental

was found over a wide range of clinical concentrations. At a total concentration of 10 $\mu\text{g/ml}$, 89.3 per cent binding (10.7 per cent unbound) was found by ultrafiltration and 88.5 per cent binding (11.5 per cent unbound) by equilibrium dialysis. Discrepancies between the results of this study, that of Koch-Weser and Sellers,⁶ and the ¹⁴C results of Dayton *et al.*,⁵ and the spectrophotometric and spectrofluorometric work of others are not readily explained, but may be due to incomplete extraction of thiopental or interference from proteins and metabolites of thiopental with the spectrophotometric and spectrofluorometric assays.

References

1. Kamanen G, Osiewicz R, Sunshine I: Barbiturate analysis—a current assessment. *J Chromatogr Sci* 10:283–287, 1972
2. Brodie BB, Mark LC, Papper EM, Lief PA, et al: The fate of thiopental in man and a method for its estimation in biological material. *J Pharmacol Exp Ther* 98: 85–96, 1950
3. Ghoneim MM, Pandya H: Plasma protein binding of thiopental in patients with impaired renal or hepatic function. *ANESTHESIOLOGY* 42:545–549, 1975
4. Mark LC, Perel JM, Brand L, et al: Studies with thiohexital, an anesthetic barbiturate metabolized with unusual rapidity in man. *ANESTHESIOLOGY* 29:1159–1166, 1968
5. Dayton PG, Perel JM, Landrau MA, et al: The relationship between binding of thiopental to plasma and its distribution into adipose tissue in man, as measured by a spectrofluorometric method. *Biochem Pharmacol* 16:2321–2326, 1967
6. Koch-Weser J, Sellers EM: Binding of drug to serum albumin (in two parts). *N Engl J Med* 294:311–316 and 526–530, 1976

Hepatic Function and Anesthesia

HALOTHANE AND HEPATIC FUNCTION

Thirty-nine patients with uterine cervical carcinoma underwent repeated radium insertion. They were randomly divided into two groups. One received halothane for all procedures (18 patients); the other received neither halothane nor methoxyflurane (21 patients). SGPT levels were measured the day before each anesthetic; patients received four anesthetics within four weeks. If SGPT was greater than 100 IU/l, further tests of hepatic function were performed. The two groups were comparable prior to the institution of treatment and had no prior clinical evidence of hepatic disease. In no control patient was any SGPT level greater than 100 IU/l recorded. Four of the patients receiving halothane developed SGPT elevations to more than 100 IU/l; three of them had been exposed to halo-

thane on three occasions. No abnormality in any other test of hepatic function was observed. (Trowell J, Peto R, Smith AC: *Controlled Trial of Repeated Halothane Anaesthetics in Patients with Carcinoma of the Uterine Cervix Treated with Radium*. *Lancet* 1: 821–824, 1975.) ABSTRACTER'S COMMENT: Alterations of post-anesthetic hepatic function produced by halothane have been observed even when operation is not performed (Stevens WC, Eger EI, Joas TA, et al, *Canad Anaesth Soc J*: 20: 357–368, 1973). These authors felt that these resulted from the action of halothane metabolites rather than the drug itself. As yet, there is no convincing evidence that these minor changes of hepatic function bear any relationship to "fulminant halothane hepatitis."