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Note

Simple, rapid and sensitive reversed-phase high-performance liquid chromatographic method for thiopental and pentobarbital determination in plasma and brain tissue

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Barbiturates such as pentobarbital and thiopental are used clinically to treat intraoperative intracranial hypertension [1], metabolic coma [2], head injury [3] and cerebral ischemia [4,5]. Because barbiturates complicate determination of both the origin and depth of coma, are used in long-term therapy, and are potent circulatory and respiratory depressants, a rapid and accurate method of quantitating plasma levels is needed to guide therapy. Furthermore, because many of the questions on the therapeutic use of barbiturates such as optimal dose and duration of administration are unresolved, pursuit of these questions in animal models would be greatly facilitated by a method enabling rapid and accurate quantitation of barbiturate levels in brain tissue and blood.

Quantitation of pentobarbital and thiopental in biological fluids have been done mainly by gas—liquid chromatography (GLC) after extensive extraction procedures and derivatization [6—11]. Poor reproducibility and sensitivity usually resulted because of degradation at high temperatures, incomplete derivatization, reversible or irreversible absorption onto the packing materials and peak tailing.

Two high-performance liquid chromatographic (HPLC) methods have been reported for the determination of thiopental in plasma [12,13]. The first method [12] of direct injection of plasma samples into the column is not applicable for tissue samples. It has the advantage of simplicity but suffers the disadvantage of rapid loss of column efficiency. The use of a precolumn prolonged the life of the analytical column, but reproducibility was poor. The second method [13] measures plasma concentrations of the thiopental and lidocaine by a multiple-step extraction procedure. This method has low

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sensitivity and strong interference by plasma constituents. In neither method was quantitation of pentobarbital mentioned.

Our HPLC assay for pentobarbital and thiopental has several advantages over GLC and other HPLC methods. A simple one-step extraction is used to separate the barbiturates from biological fluids and tissues, and the reconstituted sample is directly injected into the chromatograph. Both pentobarbital and thiopental were quantitated with excellent sensitivity and reproducibility.

EXPERIMENTAL

Reagents and materials

Sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL, U.S.A.) and thiopental (Pentothal, Abbott Laboratories) were used. Solvents were of HPLC grade and other chemicals were analytical reagent grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Stock solutions of the barbiturates (1.0 mg/ml) were prepared in 75% methanol. Phosphate buffer was prepared by titrating 0.01 *M* potassium hydrogen phosphate with 0.01 *M* potassium dihydrogen phosphate to pH 7.8.

Apparatus

A Waters Assoc. (Waters Assoc., Milford, MA, U.S.A.) high-performance liquid chromatograph equipped with a constant-flow pump (Model 6000A), a loop-type injector (Model U6K), and a fixed wavelength (254 nm) UV detector (Model 440) was used. A stainless-steel column (30 cm × 3.9 mm) packed with fully porous 10-μm silica particles with a chemically bonded monomolecular layer of octadecylsilane (μBondapak C_{18}) was utilized for barbiturate separation. A microprocessor (Model C-R1A Chrompatopac, Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) was used for peak area integration and calculations.

Chromatographic conditions

The mobile phase consisted of 0.01 *M* phosphate buffer—acetonitrile—tetrahydrofuran (THF) (78:22:4). A flow-rate of 2.0 ml/min was established at about 14 MPa.

Preparation of plasma standards

Various amounts of thiopental and pentobarbital stock solutions were added to human plasma. The concentration ranges of the standards were from 0—30 μg/ml of thiopental and 0—50 μg/ml of pentobarbital. Thiopental served as the internal standard for pentobarbital determination while pentobarbital was the internal standard for the thiopental assay.

Extraction and HPLC assay

Plasma samples. Two μg of thiopental or 4 μg of pentobarbital internal standard in 0.2 ml of plasma, 0.1 ml of 1 *N* hydrochloric acid and 2 ml of chloroform were placed into a 15-ml screw-capped centrifuge tube. The tubes were agitated on a mechanical shaker (Burrell wrist-action shaker, Burrell

Corporation, Pittsburgh, PA, U.S.A.) for 15 min, then centrifuged at 500 *g* for 10 min. The chloroform layer was transferred to a conical test tube, evaporated to dryness at 40°C under a stream of nitrogen, and reconstituted with 200 μl of the mobile phase. A 30-μl aliquot was injected into the HPLC system.

Brain samples. Whole rat brain was weighed and homogenized (Polytron, Brinkman Instruments, Westbury, NY, U.S.A.) in 4 ml distilled water per gram of tissue. Pentobarbital and thiopental (5—20 μg) were added to 4 ml of the homogenate. After mixing with 1 ml of 1 *N* hydrochloric acid, it was extracted, dried under nitrogen and redissolved in 1 ml of mobile phase. A 30-μl aliquot was injected into the HPLC system.

Recovery. The recovery of the barbiturates from plasma and brain tissue was determined by comparing chromatographic peak areas of pentobarbital and thiopental in plasma and brain samples with peak areas obtained by direct injection of equal amounts of the drugs in solvent.

Validation of the assay method for plasma samples

Known amounts of the barbiturates were added to aliquots of plasma to obtain various final concentrations. The samples were assayed in triplicate and the coefficients of variation and relative errors were calculated.

RESULTS AND DISCUSSION

The calibration curves were constructed by plotting the peak area ratios of thiopental or pentobarbital to their internal standards against known concentrations of thiopental and pentobarbital. Excellent linearity was observed ($r > 0.996$) for both barbiturates, but the linear range for thiopental (0—20 μg/ml) was less than for pentobarbital (0—50 μg/ml). Standard curves were constructed by least-square regression for calculation of unknown concentrations.

Under the assay conditions, baseline resolution of pentobarbital and thiopental were obtained with retention times of 9.5 and 12.0 min, respectively (Fig. 1c). If the amount of THF was reduced or eliminated from the mobile phase, the resolution of both barbiturates was less (Fig. 1b) or there was no separation at all (Fig. 1a). THF is thought to form intermolecular hydrogen bonding with the underivatized imino or oxo functional groups of the barbiturates and hence shield the hydrophilic sites. The formation of the less hydrophilic complex by THF greatly increased the resolution and decreased peak tailing.

In plasma, more than 93% of the pentobarbital was extracted in contrast to 82% for thiopental (Table I). The smaller amount of thiopental extracted could be due to the higher lipophilicity or affinity of thiopental to the plasma constituent. However, the ratio of the recovery of these two barbiturates was constant at these concentrations which is essential for using each as the internal standard. Recovery of both barbiturates was substantially less in brain tissue ranging between 46 and 49% for pentobarbital and between 40 and 44% for thiopental. This was probably due to the loss of chloroform by gel formation of the chloroform—homogenate mixture. However, the ratios of percent recovery were similar to that in plasma.

Determination of the precision and accuracy for determination in plasma

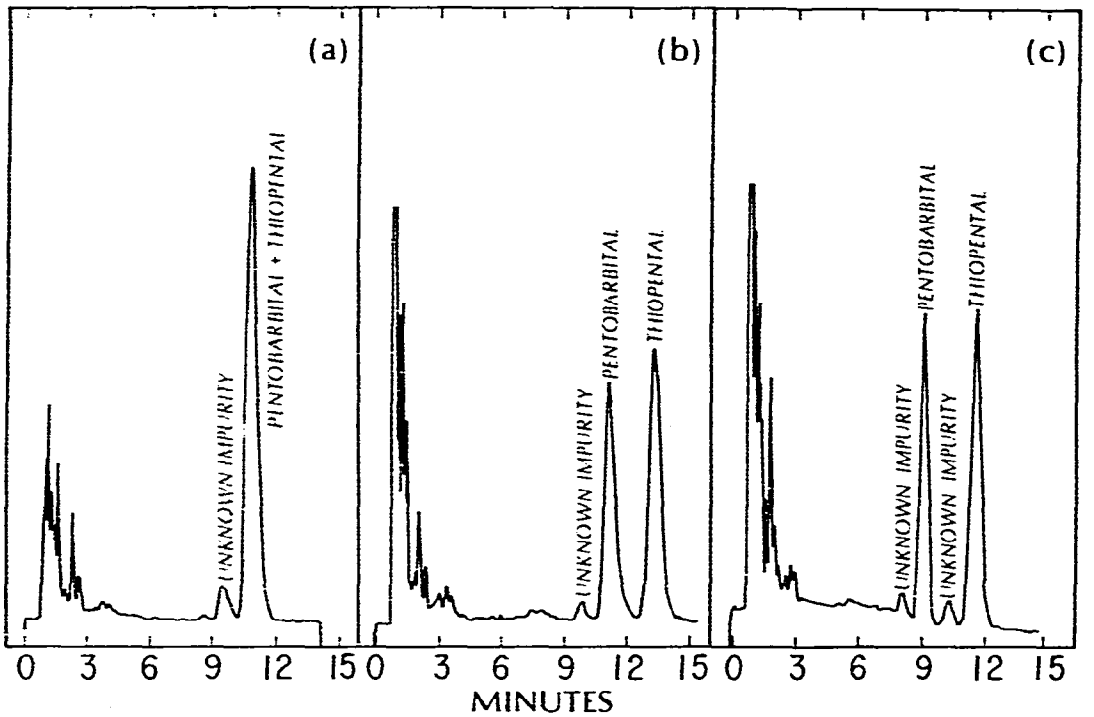

Fig. 1. Chromatograms obtained from plasma samples. The concentrations of pentobarbital and thiopental were equivalent to 20 and 10 μg per ml of plasma. A 30-μl aliquot of the final reconstituted solution was injected. The effluent was detected at 254 nm and 0.01 a.u.f.s. The mobile phases were 0.01 *M* phosphate buffer (pH 7.8)—acetonitrile—THF at ratios of: (a) 78:22:0; (b) 78:22:2, and (c) 78:22:4.

TABLE I

RECOVERY OF THIOPENTAL AND PENTOBARBITAL FROM PLASMA AND BRAIN TISSUE

In all cases $n = 3$. Values given as mean (S.D.).

Concentration (μg/ml)	Pentobarbital	Thiopental	Ratio*
Percent recovery from plasma			
5.0	94.2 (9.1)	83.3 (8.2)	0.88
10.0	93.1 (7.0)	81.9 (7.5)	0.88
20.0	93.4 (5.7)	81.8 (1.7)	0.87
Percent recovery from brain			
5.0	48.9 (1.7)	43.8 (2.0)	0.89
10.0	46.3 (3.6)	40.0 (2.6)	0.86
20.0	47.9 (2.4)	41.2 (3.6)	0.86

*Mean percent of thiopental:pentobarbital.

reveal an overall coefficient of variation of 4.3% for pentobarbital (Table II) and 4.0% for the thiopental (Table III) and average relative errors of 4.7% and 5.3%, respectively.

TABLE II

PRECISION AND ACCURACY OF HPLC ASSAY OF PENTOBARBITAL IN PLASMA

In all cases $n = 3$.

Actual concentration (μg/ml)	Measured concentration [μg/ml, mean (S.D.)]	Coefficient of variation	Relative error (%)
3.80	3.98 (0.045)	1.1	4.7
6.82	7.12 (0.184)	2.6	4.4
14.5	14.0 (0.577)	4.1	3.7
23.9	22.2 (1.71)	7.7	7.4
37.7	37.8 (3.04)	8.0	6.1
51.2	51.9 (1.10)	2.1	2.0
	(overall)	(4.3)	(4.7)

TABLE III

PRECISION AND ACCURACY OF HPLC ASSAY OF THIOPENTAL IN PLASMA

In all cases $n = 3$.

Actual concentration (μg/ml)	Measured concentration [μg/ml, mean (S.D.)]	Coefficient of variation	Relative error (%)
2.15	2.20 (0.145)	6.6	5.4
4.68	4.54 (0.104)	2.3	2.8
9.71	9.83 (0.484)	4.9	4.1
14.5	14.5 (0.737)	5.1	3.9
19.2	19.2 (0.306)	1.6	1.2
28.5	24.3 (0.902)	3.7	14.6
	(overall)	(4.0)	(5.3)

The sensitivity for both barbiturates was about 0.1 μg/ml in plasma and 0.2 μg/g in brain tissue. Because only a small portion of the sample was used for each determination, the sensitivity could be greatly improved by increasing sample size (by using 1 ml of plasma instead of 0.2 ml) or by reconstituting the sample residue in a smaller volume of mobile phase solvent. Since there was no interference by using the crude plasma extract, we estimate that a 10- to 20-fold increase in sensitivity could be obtained. Sensitivity could also be improved by using detector wavelengths of maximum absorbancy. Absorbance for thiopental was higher at 300 and 200 nm than at 254 nm (Fig. 2). UV absorption by pentobarbital was greatly increased by lowering the wavelength to less than 240 nm. Thus, with the use of the variable-wavelength UV detector a higher sensitivity may be obtainable.

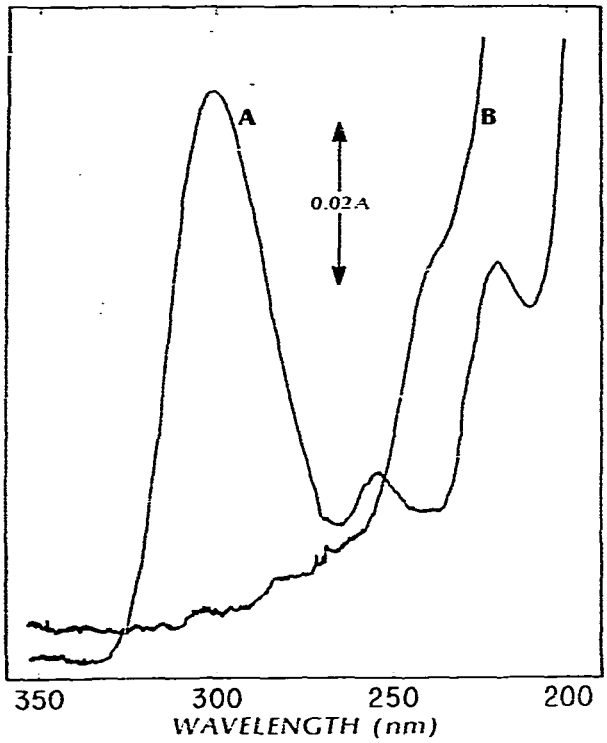

Fig. 2. UV absorption spectra of thiopental (A) and pentobarbital (B). The concentrations were 1.0 and 2.5 μg of thiopental and pentobarbital per ml of mixed solvent of 0.01 *M* phosphate buffer—acetonitrile—THF (78:22:4).

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