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Note

Determination of sodium pentobarbital and either sodium methohexital or sodium thiopental in plasma by high-performance liquid chromatography with ultraviolet detection

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Sodium pentobarbital [(5-ethyl-5-(1-methylbutyl)barbituric acid sodium salt] is a commonly used anesthetic when conducting experiments in dogs [1] but has the disadvantage that full recovery requires 24–48 h. Sodium thiopental [5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid sodium salt] and sodium methohexital [*α-dl*-1-methyl-5-allyl-5-(1-methyl-2-pentynyl)barbituric acid sodium salt] are barbiturates used for the induction of general anesthesia in humans [2] and may have pharmacokinetic properties that will make them useful in providing canine anesthesia that is superior to that provided by sodium pentobarbital when they are administered by a pharmacokinetically designed intravenous infusion.

Many methods have been developed to measure the barbiturates by high-performance liquid chromatography (HPLC). One method is able to measure both methohexital and thiopental with good sensitivity (0.125 and 0.250 μg/ml, respectively) after a relatively simple extraction of 1 ml of plasma but it has a poor peak-to-noise ratio because of the low detection wavelength (195 nm) and it does not seek to detect or measure pentobarbital [3]. Another method for thiopental has reasonable sensitivity (0.5 μg/ml), but the column rapidly loses efficiency, because plasma is injected directly into the HPLC system, and it cannot detect pentobarbital because detection is at 280 nm [4]. A micromethod for measuring thiopental using a simple preparation of 50 μl of plasma also reports reasonable sensitivity (0.5 μg/ml), but pentobarbital must be measured by another technique because detection is also at 280 nm [5].

A sensitive technique for the determination of plasma thiopental concentrations that uses a simple, one-step extraction has been reported [6], but it has two

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disadvantages. This method uses pentobarbital as an internal standard; pentobarbital should be resolved from both thiopental and the internal standard because it is either a metabolite of thiopental [7,8] or an artifact of its extraction from plasma [9]. In addition this method resolves unknown impurities from both the pentobarbital peak and the thiopental peak; the unknown impurities may be the isomers of these drugs [10,11], and at least the thiopental isomer has the same pharmacokinetic profile and anesthetic potency as thiopental, making resolution unnecessary [11].

Several methods are able to separate and simultaneously quantitate plasma pentobarbital and thiopental concentrations. One method employs a large volume of plasma (2 ml), uses a laborious extraction technique, and has limited sensitivity (1 μg/ml for thiopental and 2 μg/ml for pentobarbital) [12] while the other uses a different wavelength for each of the barbiturates (212 nm for pentobarbital and 284 nm for thiopental) to achieve a sensitivity of 0.5 μg/ml [13].

It was the purpose of the present study to develop, for purposes of pharmacokinetic analyses, simple and sensitive HPLC techniques for the determination of plasma concentrations of sodium pentobarbital, sodium methohexital, and sodium thiopental. The mobile phase used in this method is based on that of Shiu and Nemoto [6]. An important feature of this technique is the facile isolation of the barbiturates from plasma with inexpensive disposable solid-phase extraction columns which is a modification of that previously described for the muscle relaxants D-tubocurarine chloride and metocurine iodide [14]; the chemically gentle nature of this technique minimizes possible artifactual formation of pentobarbital from thiopental previously described for a liquid–liquid extraction technique [9]. In addition, these techniques do not resolve the isomers of pentobarbital and thiopental because such resolution is both unnecessary and undesirable [11]. Furthermore, the sensitivity obtained with this technique by monitoring UV absorption at 254 nm is adequate even for pharmacokinetic studies of standard doses of these drugs, making dual-wavelength UV absorption monitoring to increase sensitivity unnecessary.

EXPERIMENTAL

Reagents

The sodium salts of pentobarbital (Sigma, St. Louis, MO, U.S.A.), methohexital (Eli Lilly, Indianapolis, IN, U.S.A.), thiopental (Abbott Labs., North Chicago, IL, U.S.A.), and secobarbital [5-allyl-5-(1-methylbutyl)barbituric acid sodium salt] (Sigma) reference standards were used after drying at room temperature in a vacuum desiccator. Methanol and tetrahydrofuran were HPLC grade (American Burdick & Jackson, Muskegon, MI, U.S.A.). All other chemicals were reagent grade.

Sample collection and preparation

Blood samples (5 ml for pentobarbital or methohexital but only 3 ml for thiopental because less plasma is needed to achieve the desired sensitivity) were obtained by syringe through an arterial catheter, previously inserted in a femoral

artery for blood pressure monitoring, and transferred to Vacutainer (Becton Dickinson, Rutherford, NJ, U.S.A.) collection tubes containing sodium heparin. The plasma samples were removed after centrifugation of the blood for 10 min at 1800 *g* and transferred to polypropylene test tubes. Thiopental plasma samples were extracted in duplicate on the day they were obtained; reconstituted extracts were stored at 4 °C until analyzed. Pentobarbital and methohexital plasma samples were stored at −30 °C until extracted in duplicate.

Sample extraction

Pentobarbital, methohexital, and thiopental were extracted from plasma using Bond-Elut 100-mg C_{18} solid-phase extraction columns in conjunction with the Vac-Elut ten-place vacuum manifold (Analytichem International, Harbor City, CA, U.S.A.). The columns were conditioned by sequential flushes (under vacuum) of 2 column volumes of methanol, 2 volumes of water, and 1 volume of pH 5.59 Sörensen's 0.1 *M* phosphate buffer. Plasma samples of 0.5 ml (0.2 ml for thiopental) were then added to the columns along with 10 μl of the internal standard solution, 1.0 mg sodium secobarbital per ml reagent alcohol, and the vacuum was reapplied. The columns were next washed, under vacuum, with 2 volumes of pH 5.59 Sörensen's 0.1 *M* phosphate buffer and 1 volume of water (with 1 volume of water, no buffer, for thiopental). Subsequent application of 500 μl of methanol (250 μl for thiopental) under vacuum, eluted the barbiturates from the column into 1.5-ml polypropylene micro-centrifuge tubes (American Scientific Products, McGaw Park, IL, U.S.A.). The vacuum was then maintained no longer than required for the samples to dry (approximately 30 min) to minimize loss of drug. Prior to injection into the HPLC system the samples were reconstituted with 50 μl of methanol and mixed on a vortex mixer.

Chromatographic apparatus and conditions

The HPLC system consisted of Waters Assoc. (Milford, MA, U.S.A.) Model M-45 solvent delivery system, Model U6K universal liquid chromatograph injector, RCM-100 radial compression separation system with a Radial-Pak 10-μm C_8 (10 cm×8 mm) cartridge and a Guard-Pak 10-μm C_{18} precolumn insert, and Model 440 absorbance detector with a 254-nm wavelength kit. Pentobarbital, methohexital, and thiopental were eluted isocratically at ambient temperature and at 2.5 ml/min with a pH 7.73 Sörensen's 0.1 *M* phosphate buffer–methanol–tetrahydrofuran (13:7:4) mobile phase that had been filtered through a 0.22-μm Durapore Filter (Waters Assoc.). The chromatograms were recorded, the peaks were identified and integrated, and the concentrations were reported on the basis of the internal standard area ratio method by the 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Evaluation of the methods

The linearity, accuracy, and precision of the assays were assessed by the measurement of the sodium pentobarbital, the sodium methohexital, or the sodium thiopental concentrations of replicate plasma standards over a period of several weeks. The plasma standards were made by adding known amounts of the bar-

biturates from ethanolic stock solutions (the sodium thiopental stock solutions were always freshly prepared from the 5.0 mg/ml ethanolic stock) to blank normal human plasma. These plasma standards contained 0.25 (sodium thiopental only), 0.50, 1.00, 2.50, 5.00, 10.00, 25.00, 50.00 or 100.00 μg/ml of sodium pentobarbital, sodium methohexital or sodium thiopental. Recovery was evaluated by comparing the peak areas of 1.00, 10.00, and 100.00 μg/ml extracted plasma standards to those of the standard stock solutions.

Canine study

The usefulness of these methods for pharmacokinetic studies was evaluated in three male mongrel dogs. The pentobarbital study dog was anesthetized initially with intravenous ketamine (Ketalar Hydrochloride, Parke-Davis, Morris Plains, NJ, U.S.A.), 10 mg/kg, and received sodium pentobarbital (Nembutal Sodium, Abbott Labs.), 30 mg/kg intravenously, as it recovered from the ketamine anesthesia; the pentobarbital anesthesia was supplemented at 360 min with a single dose of ketamine (3.3 mg/kg intravenously). The methohexital study dog was anesthetized initially with intramuscular ketamine, 10 mg/kg, and intravenous ketamine, 15 mg/kg, and received sodium methohexital (Brevital Sodium, Eli Lilly), 30 mg/kg intravenously, as it recovered from the ketamine anesthesia; anesthesia was maintained with a pentobarbital infusion based on previously described pharmacokinetics [15] and designed to maintain plasma concentrations at approximately 30 μg/ml. The thiopental study dog was anesthetized throughout the study with the pharmacokinetically designed sodium pentobarbital infusion, and sodium thiopental (Pentothal Sodium, Abbott Labs.), 10 mg/kg intravenously, was administered 2 h into the sodium pentobarbital infusion. The three dogs were ventilated via an endotracheal tube while they were anesthetized by the barbiturates and received supplemental hydration with an intravenous crystalloid solution (American McGaw, Irvine, CA, U.S.A.). Arterial blood samples were obtained before and frequently after the rapid intravenous administration of either sodium pentobarbital, sodium methohexital or sodium thiopental.

RESULTS AND DISCUSSION

HPLC profiles of sodium pentobarbital, sodium methohexital, and sodium thiopental are shown in Fig. 1. Neither ketamine nor the pentobarbital administered concomitantly with the other drugs was found to interfere with the assay.

The accuracy and precision of the HPLC techniques for the measurement of sodium pentobarbital, sodium methohexital, and sodium thiopental are summarized in Tables I, II, and III, respectively. Linear regression analyses of the standard barbiturate concentrations from 0.50 to 100.0 μg/ml (0.25–100.0 μg/ml for sodium thiopental) versus barbiturate/internal standard area ratios verified the linearities of the sodium pentobarbital ($r=0.999$; $y=20.45x+0.05$), the sodium methohexital ($r=0.999$; $y=20.10x-0.09$), and the sodium thiopental ($r=0.999$; $y=15.61x-0.02$) standard curves. When sodium pentobarbital is measured simultaneously with sodium thiopental the lower limit of detection is 1.25 μg/ml when 200 μl rather than 500 μl of plasma are used but the accuracy and precision

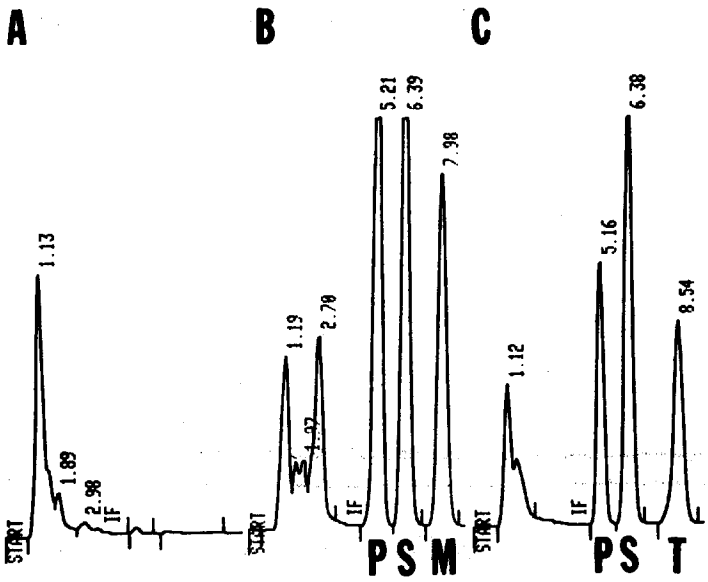

Fig. 1. Chromatograms of sodium pentobarbital (P), sodium methohexital (M), and sodium thiopental (T) extracted from plasma with the internal standard, sodium secobarbital (S). The ordinate is response and the abscissa is time (min). The retention times for the peaks are indicated on the chromatograms. (A) Blank plasma; (B) plasma of a dog obtained 30 min after the administration of 30 mg/kg sodium methohexital with anesthesia being provided by an infusion of sodium pentobarbital; (C) plasma of a dog obtained 30 min after the administration of 10 mg/kg sodium thiopental with anesthesia being provided by an infusion of sodium pentobarbital.

for this assay are unchanged. The average recoveries for six replicate samples at 1.00, 10.00, and 100.0 μg/ml were 94.5, 94.7, and 91.8%, respectively, for sodium pentobarbital, 83.6, 83.4, and 83.0%, respectively, for sodium methohexital, and 84.4, 85.7, and 88.6%, respectively, for sodium thiopental. The average recovery for sodium secobarbital, the internal standard, was 94.3% ($n=18$).

TABLE I

ACCURACY AND PRECISION FOR THE PLASMA SODIUM PENTOBARBITAL ASSAY ($n=6$)

Concentration added (μg/ml)	Concentration measured (mean ± S.D.) (μg/ml)	Mean error (μg/ml)	Relative error (%)	Coefficient of variation (%)
0.50	0.547 ± 0.036	0.047	9.4	6.6
1.00	1.056 ± 0.056	0.056	5.6	5.3
2.50	2.542 ± 0.119	0.042	1.7	4.7
5.00	5.152 ± 0.212	0.152	3.0	4.1
10.00	10.22 ± 0.43	0.22	2.2	4.2
25.00	25.22 ± 0.82	0.22	0.8	3.3
50.00	48.82 ± 0.87	1.18	2.4	1.8
100.00	100.4 ± 2.2	0.4	0.4	2.2

TABLE II

ACCURACY AND PRECISION FOR THE PLASMA SODIUM METHOHEXITAL ASSAY ($n=6$)

Concentration added (μg/ml)	Concentration measured (mean ± S.D.) (μg/ml)	Mean error (μg/ml)	Relative error (%)	Coefficient of variation (%)
0.50	0.543 ± 0.038	0.043	8.6	7.0
1.00	1.049 ± 0.038	0.049	4.9	3.6
2.50	2.575 ± 0.096	0.075	3.0	3.7
5.00	5.196 ± 0.184	0.196	3.9	3.5
10.00	10.28 ± 0.50	0.28	2.8	4.9
25.00	24.97 ± 0.80	0.03	0.1	3.2
50.00	49.02 ± 0.92	0.98	2.0	1.9
100.00	100.3 ± 4.0	0.3	0.3	4.0

In the initial stages of the methods development the dried plasma barbiturate extracts were reconstituted with 50 μl of mobile phase. It was soon discovered that thiopental was unstable even in this medium; when 10 μg/ml standard extracts were reconstituted with mobile phase and injected every 0.5 h for up to 4.5 h the measured sodium thiopental concentration decreased by more than 10% per hour ($r=-0.934; y=-1.053x+9.545$) but no similar decrease was observed when the extracts were reconstituted with methanol ($r=0.069; y=-0.017x+10.219$). Although no similar instability was observed for the other barbiturates, methanol was subsequently used for reconstituting all of the plasma barbiturate extracts.

Column carry-over is a common problem associated with the HPLC analysis of thiol-containing compounds [16] and the slight carry-over observed with thiopental in this assay was eliminated by adding no less than 5 ng/ml sodium thiopental to the mobile phase.

Plasma sodium pentobarbital, sodium methohexital, and sodium thiopental versus time relationships in the animals studied are illustrated in Fig. 2. This

TABLE III

ACCURACY AND PRECISION FOR THE PLASMA SODIUM THIOPENTAL ASSAY ($n=6$)

Concentration added (μg/ml)	Concentration measured (mean ± S.D.) (μg/ml)	Mean error (μg/ml)	Relative error (%)	Coefficient of variation (%)
0.25	0.226 ± 0.021	0.024	9.6	9.3
0.50	0.478 ± 0.023	0.022	4.4	4.8
1.00	0.971 ± 0.034	0.029	2.9	3.5
2.50	2.480 ± 0.026	0.020	0.8	1.0
5.00	4.993 ± 0.160	0.007	0.1	3.2
10.00	9.93 ± 0.42	0.07	0.7	4.2
25.00	25.52 ± 1.09	0.52	2.1	4.3
50.00	50.07 ± 1.09	0.07	0.1	2.2
100.00	99.6 ± 5.2	0.4	0.4	5.2

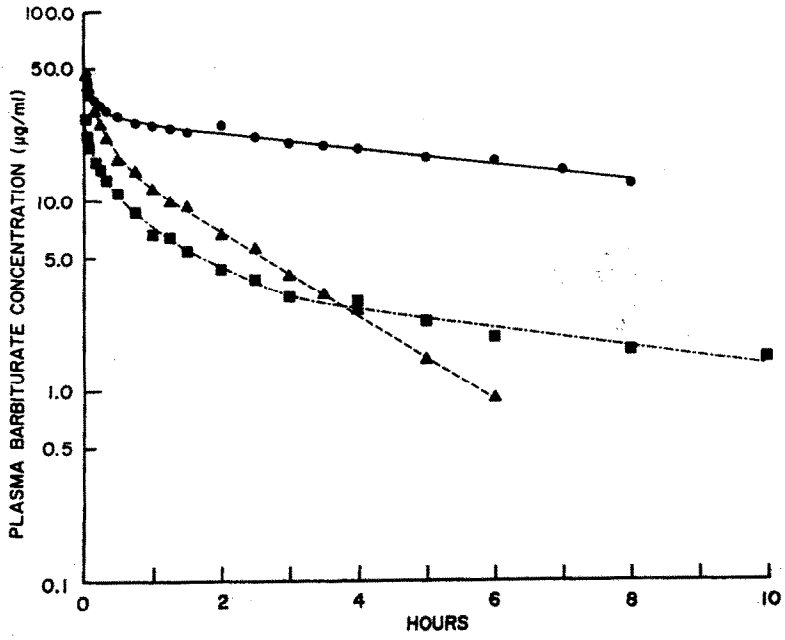

Fig. 2. Plasma barbiturate versus time relationships after the administration of 30 mg/kg sodium pentobarbital (●), 30 mg/kg sodium methohexital (▲), and 10 mg/kg sodium thiopental (■) to 31-, 33-, and 24-kg dogs, respectively. The lines are computer-derived non-linear least-squares regression lines through the measured plasma concentrations at the various times.

illustrates the differences in the plasma concentration versus time relationships between the ultrashort-acting barbiturates methohexital and thiopental and the intermediate-acting pentobarbital, as well as differences between the high elimination clearance methohexital and the low elimination clearance barbiturates pentobarbital and thiopental. The HPLC techniques described in this manuscript are, therefore, able to accurately and easily measure the plasma concentrations of these barbiturates for at least as long as is necessary to characterize their pharmacokinetics. This method might be easily adapted to measure concentrations of secobarbital, a hypnotic/sedative, using pentobarbital as the internal standard.

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