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NOTES

An Improved High-Pressure Liquid Chromatographic Assay for Secobarbital in Serum

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Abstract □ A high-pressure liquid chromatographic method for the analysis of secobarbital in serum was developed. Secobarbital was extracted from buffered serum (pH 5.5) with a solvent mix of hexane–ether–*n*-propanol. 5-(4-Methylphenyl)-5-phenylhydantoin was added as an internal standard. Separation of secobarbital and internal standard from serum constituents and other drugs was achieved on a 5-μm C-18 reversed-phase column using an acetonitrile–phosphate buffer (pH 4.4) mobile phase. The eluent was monitored at 195 nm. The sensitivity limit of the assay was ~0.02 μg/ml with 0.5 ml of serum sample. The application of this method to pharmacokinetic studies in pediatric patients was demonstrated.

Keyphrases □ Secobarbital—high-pressure liquid chromatographic assay in human serum, anticonvulsants, pharmacokinetics □ High-pressure liquid chromatography—secobarbital, anticonvulsant, pharmacokinetics, human serum □ Anticonvulsants—secobarbital, high-pressure liquid chromatographic assay in human serum, pharmacokinetics □ Pharmacokinetics—secobarbital, high-pressure liquid chromatographic assay in human serum, anticonvulsant

Secobarbital is used primarily as a hypnotic agent. Like many other barbiturates, when administered in anesthetic doses it is an effective anticonvulsant (1). Rectal secobarbital is prescribed for the emergency home treatment of prolonged seizures in poorly controlled epileptic children (2). In an attempt to study the bioavailability of rectally administered secobarbital in pediatric patients, a sensitive method for quantitating the drug in serum was required.

A number of analytical procedures for determining secobarbital in serum have been described in the literature. These include spectrophotometry (3), gas-liquid chromatography (GLC) (4–8), and high-pressure liquid chromatography (HPLC) (9, 10). However, many of these existing procedures are aimed at detecting high concentrations (>5 μg/ml) of secobarbital for screening purposes related to drug abuse. The more sensitive methods such as GLC with electron capture detection require tedious sample clean up and derivatization procedure prior to analysis. Also, with many of these methods, the problem of interference from other drugs and their metabolites in serum has not been evaluated. This report describes an improved HPLC method that permits the determination of submicrogram quantities of secobarbital in small volumes of serum in the presence of various antiepileptic drugs.

EXPERIMENTAL

Reagents and Chemicals—Secobarbital[1] and 5-(4-methylphenyl)-5-phenylhydantoin[2] (internal standard) were used as supplied. The solvents used for extraction and chromatography were all of HPLC grade[3,4]. All chemicals were of analytical reagent grade.

A stock solution of sodium secobarbital (120 μg/ml) and the internal standard (7 μg/ml) were prepared in water. The phosphate buffer component of the mobile phase was prepared by adding 300 μl of 1.0 *M* KH_2PO_4 and 50 μl of 0.9 *M* H_3PO_4 to 1800 ml of water (pH 4.4). For the extraction procedure, a pH 5.5 acetate buffer consisting of 0.01 *M* sodium acetate–0.01 *M* acetic acid (88.5:11.5, v/v) was prepared.

Apparatus and Operating Conditions—The liquid chromatograph consisted of a constant flow pump[5], a variable volume sampling valve[6], and a variable wavelength detector[7]. A 4.6-mm × 25-cm column packed with 5 μm of microporous silica chemically bonded with octadecylsilane was obtained from a commercial source[8]. The mobile phase consisting of 28% acetonitrile and 72% pH 4.4 phosphate buffer (v/v) was filtered[9]

[1] Eli Lilly and Co., Indianapolis, Ind.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Fisher Scientific Co., Rochester, NY.
[4] J. T. Baker Chemical Co., Rochester, NY.
[5] Model M6000A, Waters Associates, Milford, Mass.
[6] Model U6K, Waters Associates, Milford, Mass.
[7] Model SF770, Kratos Inc., Schoeffel Instrument Div., Westwood, N.J.
[8] Partisil 5 ODS-3, Whatman Inc., Clifton, NJ 07014.
[9] 0.5 μm FH type filter, Millipore, Bedford, Mass.

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Table I—Calibration Curve Linearity and Precision for the Concentration Range of 0.022–4.4 μg/ml (n = 8)

Day	Mean Normalized PHR[a], μg/ml	SD, μg/ml	CV, %
1	1.067	0.022	2.1
2	1.110	0.078	7.0
3	1.058	0.061	5.8
4	1.134	0.062	5.2
5	1.191	0.062	5.2
6	1.196	0.043	3.6
Mean	1.124	0.053	4.7

[a] Normalized peak height ratio defined as the peak height ratio of each secobarbital standard divided by the corresponding concentration.

Table II—Day-to-Day Precision Data Pooled From Six Separate Days of Analysis

Concentration, μg/ml	Mean Normalized PHR[a], μg/ml	SD, μg/ml	CV, %
4.4	1.150	0.004	0.3
2.2	1.093	0.044	4.0
1.1	1.132	0.071	6.3
0.55	1.138	0.100	8.8
0.275	1.127	0.107	9.5
0.11	1.091	0.047	4.3
0.055	1.128	0.109	9.7
0.022	1.138	0.057	5.0
Mean	1.125	0.067	6.0

[a] Normalized peak height ratio defined as the peak height ratio of each secobarbital standard divided by the corresponding concentration.

and degassed before use. The column was heated to 50° and the eluting solvent was pumped through the column at a flow rate of 2.8 ml/min with precolumn pressure of ~2800 psi. The column effluent was monitored at 195 nm.

Extraction—Extraction of serum samples was carried out in 15 glass centrifuge tubes sealed with polytef-lined screw caps. One milliliter of the acetate buffer and 50 μl of internal standard solution were added to 0.5 ml of serum. The mixture was agitated in a vortex mixer for 10 sec, followed by gentle shaking with 5 ml of hexane–ether–n-propanol (49:49:2, v/v/v) for 20 min. The organic and aqueous phases were separated by centrifugation at 1000×g for 5 min. The upper organic layer was transferred to a 1.5-ml polypropylene microcentrifuge tube[10]. The aqueous layer was reextracted with another 5-ml aliquot of the hexane–ether–n-propanol mixture. The second organic phase aliquot was pooled with the first and evaporated to dryness under nitrogen in a water bath at 50°. The residue was reconstituted in 300 μl of mobile phase and 50–100 μl was injected onto the column.

Standard Curves—Standardization samples were prepared by spiking blank serum (0.5 ml) with 50-μl aliquots of various dilutions of secobarbital stock solution to yield a concentration range between 0.022 and 4.4 μg/ml. Standard curves were constructed by plotting peak height ratios (secobarbital–internal standard) against the corresponding secobarbital concentration.

RESULTS AND DISCUSSION

Figure 1 shows typical chromatograms of serum samples from a pediatric patient before and after a single 5-mg/kg rectal dose of secobarbital. The drug and its internal standard were eluted at 8.5 ± 0.5 and 11.5 ± 0.7 min, respectively, under the described chromatographic conditions. Extraction recovery was evaluated by analyzing spiked serum samples. In these experiments, internal standard was added after transfer of organic phases to reduce error due to injection and chromatography. The extraction yield from serum was nearly complete and reasonably consistent (85–95%) over the chosen concentration range (0–4.4 μg/ml).

The detection limit with a 0.5-ml sample was ~0.02 μg/ml. This limit corresponded to a serum concentration that yields a peak height with a signal to noise ratio of at least 4.

Linear calibration curves were observed over the 0–4.4 μg/ml range. Calibration data obtained on six separate days are summarized in Table I. Precision was assessed by estimating the variations on normalized peak height ratios (*i.e.*, peak height ratios of each standard divided by the corresponding secobarbital concentration) (11). The coefficient of variation varied from 2.1 to 7.0%. Within-day reproducibility was also evaluated by replicate analysis of a 1.1-μg/ml serum standard. The coefficient of variation was 4.2% (n = 9). The day-to-day precision data are shown in Table II. The range of coefficient of variation for the eight concentrations was 0.3–9.7%.

Interference from endogenous materials and other drugs posed a major problem during the development of this assay. To attain good sensitivity, the loss of drug during sample preparation must be minimized. Precipitation of serum proteins with acetonitrile followed by direct injection of

[10] Walter Sarstedt Co., Princeton, N.J.

Table III—Retention Times of Anticonvulsants Detectable in the Present Chromatographic System[a]

Drugs	Retention Time, min
Ethosuximide	1.8
Primidone	2.0
Phenobarbital	2.6
Carbamazepine-10,11-epoxide	3.4
Paramethadione	3.8
Mephobarbital	7.4
Carbamazepine	7.8
Phenytoin	7.8
Secobarbital	8.5
5-(4-methylphenyl)-5-phenylhydantoin[b]	11.4

[a] Anticonvulsants that are either not detectable or not extracted include valproic acid, clonazepam, diazepam, chlorazepate, and thioridazine. [b] Internal standard.

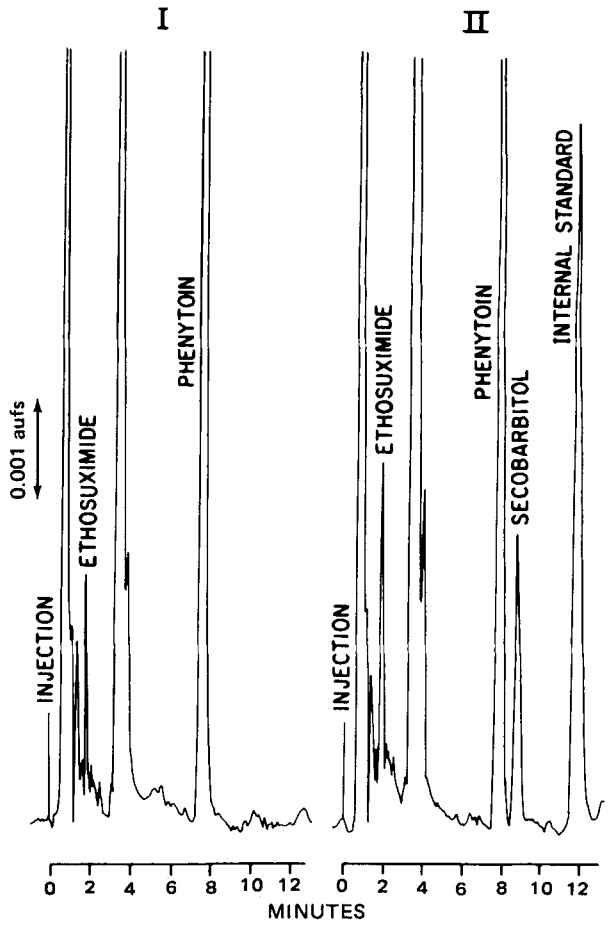

Figure 1—*Chromatogram of 0.5-ml serum extracts from a patient immediately before (I) and 90 min after (II) rectal administration of a 120-mg dose of secobarbital. The extract was reconstituted in 300 μl of eluting solvent; 50 μl was injected. The patient was a 7-year-old boy weighing 23 kg. The serum concentration of secobarbital was 0.447 μg/ml. The patient was also receiving phenytoin, ethosuximide, and clonazepam at the time of study. The observed peaks for ethosuximide and phenytoin represent serum drug concentrations of 20 and 21 μg/ml, respectively. Note the baseline separation between the secobarbital and phenytoin peaks.*

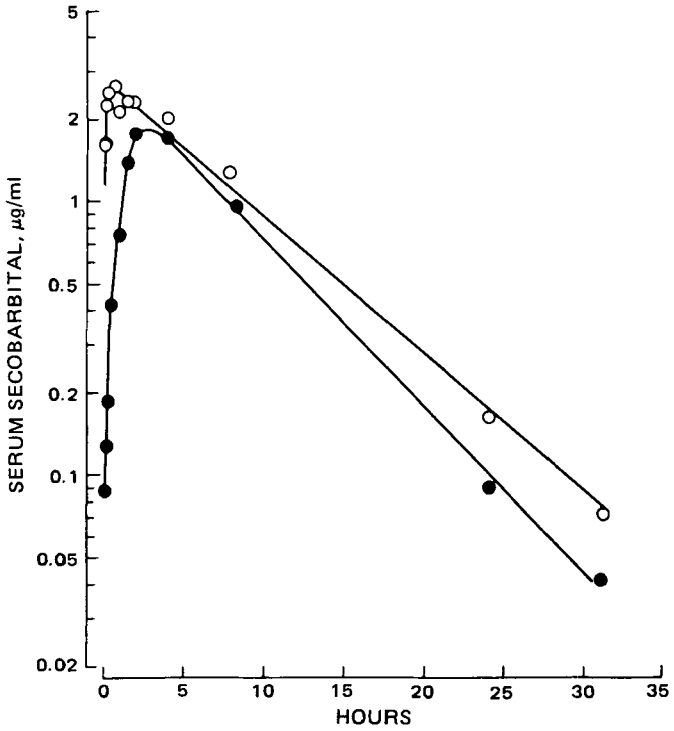

Figure 2—*Serum concentration–time course of secobarbital following separate rectal administration of a solution (○) and a suppository (●) to two 5-year-old female epileptic children with respective body weights of 17 and 19 kg.*

supernatant on the column as suggested previously (9) did produce a high recovery yield, but interference from serum constituents was observed on the chromatograms of blank samples from some patients. The problem was readily overcome by solvent extraction with hexane–ether–*n*-propranol, which provided good recovery with no interference seen in serum blanks. The extraction procedure is relatively simple, and up to 40 samples can be processed in ~1 hr.

Reversed-phase chromatography was chosen because of its versatility in separating compounds over a wide spectrum of polarity. A 10-μm reversed-phase octadecylsilane column[11] was used. Both phenytoin and carbamazepine, which coextracted with secobarbital, were eluted sufficiently close to secobarbital to hamper detection at low concentration limits. Although carbamazepine as a base can be eliminated by acid wash or back extraction, these procedures would not remove phenytoin. Also, multiple extractions reduced the recovery of secobarbital. Separation of secobarbital was achieved from both phenytoin and carbamazepine by an addition of a small quantity of tetrahydrofuran (1–2%) to the mobile phase (12). Unfortunately, this also resulted in an unstable baseline at the highest sensitivity setting (*i.e.*, 0.01 aufs) on a detector. This is probably due to the high absorptivity of tetrahydrofuran at 195 nm.

[11] μBondapak C-18, Waters Associates, Milford, Mass.

Complete resolution of secobarbital from the interfering drugs was achieved when a high-efficiency 5-μm C-18 reversed-phase column was employed. The chromatograms, as shown in Fig. 1, are taken from a patient receiving high doses of phenytoin (serum phenytoin concentration of 21 μg/ml). The phenytoin and secobarbital peaks were clearly separated.

Table III compares the retention times of secobarbital to those anticonvulsants that are detectable in our chromatographic system.

As an illustration of the applicability of our assay procedure to a single-dose pharmacokinetic study, the serum concentration–time course after rectal administration of a 5-mg/kg dose of sodium secobarbital in solution[12] and by suppository[13] in two age and weight matched epileptic children is shown in Fig. 2. The rate of absorption of secobarbital from the fatty base suppository was much slower as compared with that from the solution. Peak serum concentration was not reached until 3–4 hr after administration of the suppository, whereas peak serum concentration was achieved within 30 min with the solution preparation. The serum half-life of secobarbital in these two patients, being 6.0 and 4.9 hr, are shorter than estimates reported for adults (13).

In summary, a rapid procedure for analyzing nanogram quantities of secobarbital in serum is described. Sample preparation is minimal, and there is no interference from many other anticonvulsants.

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[12] Intravenous preparation of sodium secobarbital (seconal sodium) was diluted to a concentration of 15 mg/ml with water and administered through a rectal syringe.

[13] Seconal sodium suppositories, Eli Lilly and Co., Indianapolis, Ind.