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GAS CHROMATOGRAPHIC METHOD FOR THE MICRODETERMINATION OF BARBITURATES IN BLOOD USING A NITROGEN-SELECTIVE FLAME IONIZATION DETECTOR

BARRY H. DVORCHIK

Department of Obstetrics and Gynecology and Pharmacology, Pennsylvania State University College of Medicine, Hershey, Pa. 17033 (U.S.A.)

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SUMMARY

A rapid, quantitative gas–liquid chromatographic method for the simultaneous determination of as little as 10 ng of unmodified barbital, pentobarbital, secobarbital, and hexobarbital from whole blood is described. The method involves one extraction from whole blood into chloroform with subsequent injection into a gas chromatograph equipped with a nitrogen-sensitive flame ionization detector. This method has the advantages of small sample size, high specificity, sensitivity, and rapidity.

INTRODUCTION

The analysis of barbiturates from biological specimens by gas–liquid chromatography (GLC) is well documented[1–8]. Practically all these methods involve either lengthy extractions and clean-up techniques or require modification of the barbiturate(s). For the analysis of barbiturates from blood or plasma the methods described usually require 1–5 ml.

Anders[9] published a method for the gas chromatographic analysis of blood barbiturates which required only 100 μl of blood. This technique, however, required saturation of the column by injecting a relatively large quantity of the barbiturate(s) before the samples were to be analyzed.

The use of a nitrogen-sensitive flame ionization detector offers new possibilities in the sensitive determination of nitrogen-containing drugs in biological fluids and tissues[10]. Goudie and Burnett[11] used the nitrogen detector for the simultaneous determination of phenobarbital, primidone, and phenytoin in serum. The nitrogen detector has also been utilized in the analysis of amphetamines[12], pentazocine[13], and hexobarbital[14].

This paper describes a rapid, sensitive, and specific GLC method for the simultaneous determination of unmodified barbital, pentobarbital, secobarbital and hexobarbital from blood.

MATERIALS AND METHODS

Gas–liquid chromatography

A Hewlett-Packard Model 7620A gas chromatograph equipped with a nitrogen-sensitive flame ionization detector (NFID) was used. The columns were coiled glass tubes, 6 ft. in length with an inner diameter of 2 mm and packed with 100–120 mesh Chromosorb W AW DMCS (Supelcoport) coated with 3% SP-2250 (Supelco 01-1981) (Supelco, Bellafonte, Pa., U.S.A.). The column was preconditioned at 250° for 24 h with a helium flow-rate of about 20 ml/min. The operating conditions were: injection port and oven, 220°; detector, 400°; air flow-rate, 185 ml/min; hydrogen flow-rate, 27 ml/min; helium flow-rate, 60 ml/min. The high flow-rate for the carrier gas was required for optimum sensitivity.

Tuning of detector

To achieve optimum performance, the detector had to be tuned daily. This was achieved by injecting 1–2 μl of a test solution containing 5000 ng octadecane and 10 ng azobenzene per microliter hexane into the gas chromatograph. The distance between the collector, which contained the rubidium bromide crystal, and the flame was then changed by rotating the adjusting nut until the peak height ratio of azobenzene to octadecane was 2 (range, 10^3; attenuation, 32). Only one tuning was required on any one day.

Reagents

The barbiturates utilized in this study were: barbital (Fisher Scientific, Pittsburgh, Pa., U.S.A.), pentobarbital sodium and secobarbital sodium (Sigma, St. Louis, Mo., U.S.A.) and hexobarbital (Winthrop Labs., New York, N.Y., U.S.A.). Mephobarbital, the internal standard, was a gift from Dr. F. C. Nachod of the Sterling-Winthrop Research Institute. Sodium salts of the barbiturates were converted to their corresponding free acids by the addition of 1.0 *M* HCl to aqueous solutions of each barbiturate. The precipitated barbituric acids were separated by filtration, thoroughly air dried and stored in a desiccator under vacuum. All other reagents were of analytical grade.

Standard solutions

A standard barbiturate stock solution containing 1 mg/ml of the free acids of barbital, pentobarbital, secobarbital and hexobarbital was prepared in acetone. Working standards were prepared daily by diluting the stock solution 1:10 with acetone. Two solutions of mephobarbital in acetone, 50 μg/ml and 500 μg/ml, were also prepared. Aliquots of the working standard were pipetted into glass centrifuge tubes and evaporated to dryness at 60°, under nitrogen. The tubes were then brought up to 100 μl with the 50 μg/ml mephobarbital solution.

Standard solutions in whole blood were prepared by drying a known volume of the barbiturate working standard onto glass test tubes at 60°, under nitrogen, and dissolving the residue in 100 μl of whole blood.

Ten sets of barbiturate standards in blood (10–100 μg/ml) were prepared on the same day. Five sets were extracted with chloroform, as described below, on the same day as they were prepared and stored at 4°. The remainders were stored at

−15°. One set of each standard (*i.e.*, freshly extracted and frozen) was analyzed weekly. On the day of analysis the frozen standard was thawed and extracted with chloroform as described below.

Extraction procedure

To a 100-μl sample of whole blood in a glass tube, was added 10 μl of the 500 μg/ml mephobarbital solution and 2 ml of chloroform. The sample was shaken for 5–10 sec and then centrifuged at 100 *g* for 5 min. An aliquot of the chloroform phase (1.6 ml) was removed and transferred to clean glass-stoppered centrifuge tubes. The chloroform wsa evaporated to dryness at 60° under nitrogen; the tubes were stoppered and stored at 4° until the day of analysis. At this time the residue was brought to 100 μl with acetone.

Recovery studies

Standard solutions in whole blood were extracted as described above. The recoveries of each barbiturate at different concentrations were determined according to the following equation:

$$\% \text{ recovery} = \frac{(PHR)_B}{(PHR)_S} \times 100$$

Where $(PHR)_B$ is the peak height ratio obtained from the blood extract and $(PHR)_S$ is the peak height ratio obtained from the standard acetone solution at the same barbiturate concentration.

In vivo studies

Female Fisher rats, weighing 130–165 g, were administered either 100 mg/kg sodium hexobarbital, 30 mg/kg sodium pentobarbital, or a combination of 80 mg/kg sodium hexobarbital and 20 mg/kg sodium pentobarbital intraperitoneally. The volume of the solutions were adjusted such that 0.1 ml was given per 100 g body weight. After administration of the barbiturate(s), the rats were placed in cages and maintained at 25 ± 3° with the use of an infrared lamp. Tail vein samples were taken at predetermined times and 100-μl aliquots were pipetted into glass centrifuge tubes and frozen (−15°) until analysis. On the day of analysis the samples were thawed and extracted as described above. The half-life of the apparent terminal linear phase for the disappearance of barbiturate from the blood was determined from a least squares analysis of the log peak height ratio (barbiturate/mephobarbital) *vs.* time.

RESULTS AND DISCUSSION

Chromatograms illustrating the separation of the underivatized barbiturates at three different concentrations are shown in Fig. 1. The negative detector response which appears shortly after injection appears to be a common phenomenon associated with the nitrogen detector[14]. Retention times were: barbital, 0.5–0.67 min; pentobarbital, 0.97–1.22 min; secobarbital, 1.13–1.38 min; hexobarbital, 1.56–1.91 min; and mephobarbital, 1.88–2.25 min. Relative retention times were 0.35, 0.53, 0.63, 0.82, and 1.0, respectively. Two peaks with relative retention times of 0.37 and 0.73

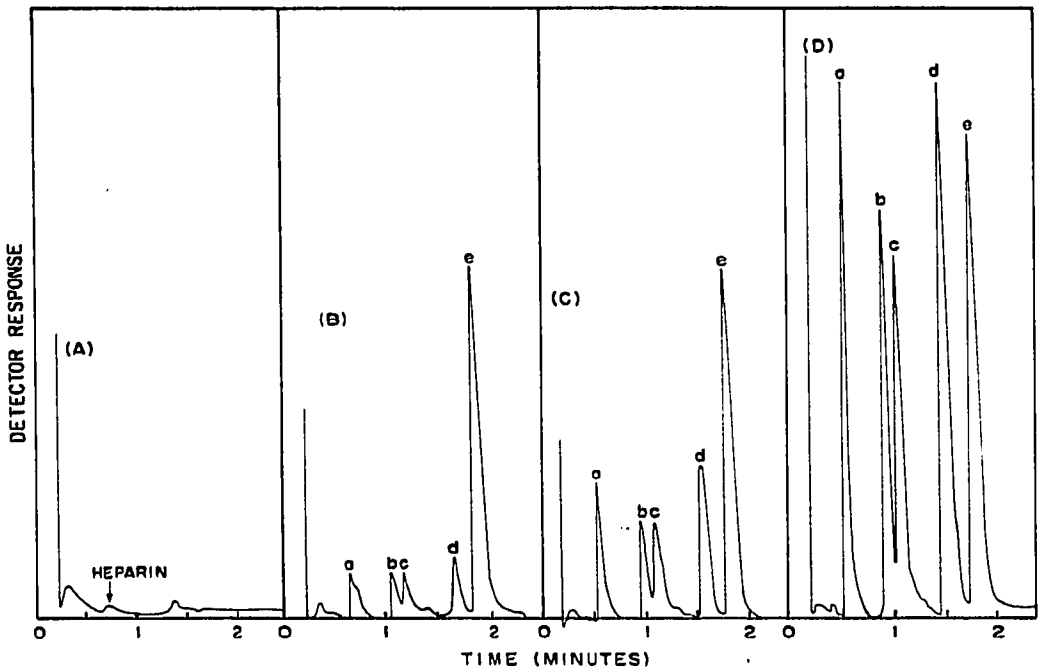

Fig. 1. Chromatograms of underivatized barbiturates extracted from whole blood. A = Control; B = 10 ng/μl; C = 20 ng/μl; D = 50 ng/μl; a = barbital; b = pentobarbital; c = secobarbital; d = hexobarbital; e = mephobarbital (internal standard, 50 ng/μl). Range, 100; attenuation, 32.

were co-extracted with the barbiturates (Fig. 1A, control blood). The first peak was found to be due to the anticoagulant (heparin).

A series of standard solutions, containing 50 μg/ml mephobarbital and from 10 to 100 μg/ml barbital, pentobarbital, secobarbital, and hexobarbital were prepared in acetone and in whole blood. Standard curves were prepared for both systems by injecting 1–2 μl of acetone standard solutions (or chloroform extract) into the gas chromatograph. The ratio of the peak height of each barbiturate to that of mephobarbital was plotted against the amount of barbiturate injected. The curves were linear over the range 10–100 ng barbiturate (Fig. 2).

If less than 10 ng of the barbiturates were applied to the column, the experimentally determined peak height ratios were always greater than those predicted from the linear regression equation obtained from the calibration curves (Table I). This apparent non-linearity has been described by others[15,16] and is due to the partial absorption of the barbiturates to the chromatographic column. It may be possible to reduce this problem somewhat by using a coarser solid support[14,17].

The stability of the detector and the effect of freezing the sample before extraction were determined by analyzing extracts from fresh and frozen barbiturate standards in whole blood. The detector response over the five-week period remained constant and regression analysis of the data indicated no significant difference between extracts of freshly prepared or frozen blood samples (Table I). We have been using this same crystal for 18 months and have not found any significant change in

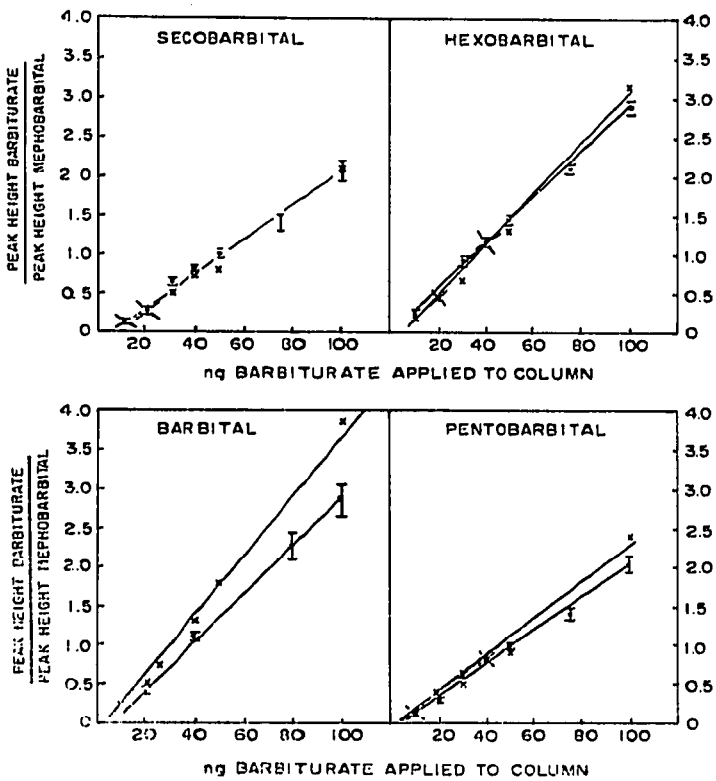

Fig. 2. Calibration curves relating peak height ratios to amount of barbiturate applied to column. × = Acetone standards; ● = blood extracts. Each point is the mean of four determinations ±S.D.

TABLE I

REGRESSION ANALYSIS OF PEAK HEIGHT RATIO *versus* BARBITURATE CONCENTRATION FOR FRESHLY EXTRACTED AND FROZEN SPIKED BLOOD SAMPLES*

Blood sample	*(Slope ±S.D.) × 100*			
	Barbital	*Pentobarbital*	*Secobarbital*	*Hexobarbital*
Fresh**	2.98 ± 0.54	2.32 ± 0.15	2.07 ± 0.16	2.64 ± 0.37
Frozen***	3.03 ± 0.63	2.27 ± 0.43	1.91 ± 0.40	2.85 ± 0.43
	Intercept			
	Barbital	*Pentobarbital*	*Secobarbital*	*Hexobarbital*
Fresh**	−0.05	−0.06	−0.04	+0.03
Frozen***	−0.07	+0.03	−0.05	−0.06

* Peak height ratio = slope × barbiturate conc. + intercept. All correlation coefficients ≥0.97.

** Average of five calibration curves, one per week.

*** Average of four calibration curves, one per week.

TABLE II

PER CENT RECOVERY OF BARBITURATES FROM BLOOD

Barbiturate	*Concentration (ng/μl)*	*Peak height ratio**		*% Recovery***	*% Mean ±S.D.*
		Standard	*Blood*		
Barbital	20	0.63	0.42	67	75 ± 7
	40	1.40	1.11	79	
	100	3.63	2.91	80	
Pentobarbital	20	0.39	0.31	80	90 ± 11
	40	0.81	0.83	102	
	100	2.30	2.05	89	
Secobarbital	20	0.29	0.31	107	103 ± 4
	40	0.80	0.82	102	
	100	2.10	2.07	99	
Hexobarbital	20	0.44	0.45	102	100 ± 5
	40	1.15	1.18	103	
	100	3.10	2.92	94	

* Average of four determinations.

** % Recovery $= \frac{(PHR)_B}{(PHR)_S} \times 100.$

peak height ratios of standard solutions or background noise that could not be corrected by cleaning the crystal with a cotton swab moistened with distilled water. We found that to maintain optimal conditions the detector should be cleaned at least twice a week.

In general it is important that the extraction gives a high yield (recovery) and is reasonably specific for the drug. One extraction with chloroform was sufficient to recover 100% of the secobarbital and hexobarbital added to whole blood (Table II). The recovery of pentobarbital was as high (90%) whereas the recovery of barbital was only 75% (Table II). These differences are explicable on the basis of the lipid solubilities of the barbiturates[18].

Hexobarbital and pentobarbital were administered intraperitoneally, either alone or in combination, to female Fisher rats. The results of a typical experiment are shown in Fig. 3. The apparent *in vivo* half-life for the disappearance of hexobarbital from the blood of these rats is in excellent agreement with that reported by Quinn *et al.*[19]; comparable data for pentobarbital are not available.

Under the conditions described in this paper we were able to measure barbiturate blood concentrations as low as 10 μg/ml (10 ng/μl). It is reasonable to assume that concentrations less than 10 μg/ml can be measured with this method by (a) increasing the sample size, (b) decreasing the volume in which the blood extract is taken up, (c) increasing the volume injected into the gas chromatograph, or (d) any combination of a, b, and c.

The simplicity of this procedure, along with its high sensitivity, specificity, and reproducibility allows for the rapid quantitative determination of barbiturates from small samples of blood. The small sample size makes this a suitable method for pharmacokinetic studies in small animals and infants and should also allow one to

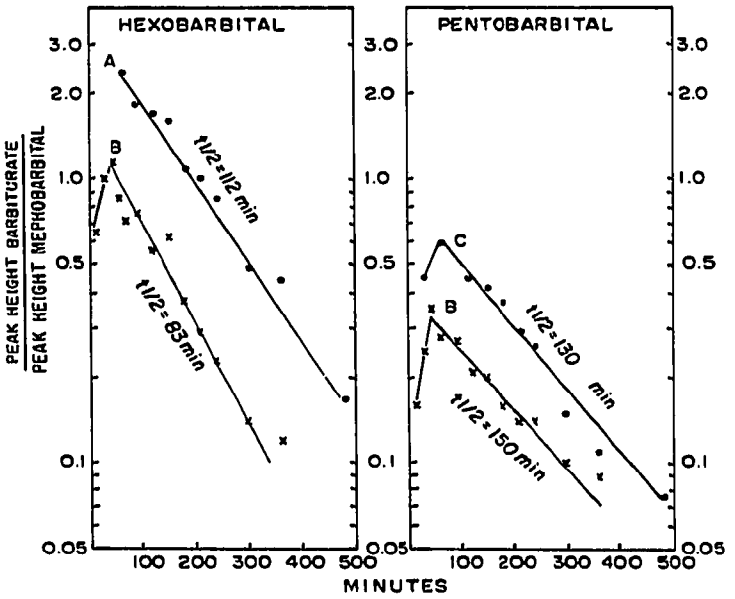

Fig. 3. Disappearance of hexobarbital and pentobarbital in the blood of female Fisher rats following intraperitoneal administration. (A) 130 g rat; 100 mg/kg Na hexobarbital. (B) 164 g rat; 80 mg/kg Na hexobarbital and 20 mg/kg Na pentobarbital. (C) 145 g rat; 30 mg/kg Na pentobarbital.

utilize capillary blood when monitoring drug levels in patients. Experiments designed to extend the method to the analysis of other drugs in blood, urine, and tissues are in progress.

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