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Micro-Sample Gas-Chromatographic Technique for the Analysis of Barbiturates[1]

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LAU, C. E., M. TANG AND J. L. FALK. *Micro-sample gas-chromatographic technique for the analysis of barbiturates.* PHARMAC. BIOCHEM. BEHAV. 11(3) 355–357, 1979.—A procedure for determining barbiturate levels in micro-samples is described. The method takes advantage of a newly available, sensitive nitrogen-phosphorus detector thereby permitting a simplified analytic procedure.

Barbiturate analysis Nitrogen-phosphorus detector Micro-method

IN RESEARCH with small laboratory animals, it is advantageous to trace changes in the blood level of a particular drug in the same animal over time. This necessitates analysis of rather small, repeated samples since larger ones could produce unwanted, cumulative effects (e.g., altered circulatory dynamics, generation of new (drug-free) plasma, or shock). Furthermore, quantification of low blood levels of barbiturates, rather than mere detection, is required in many long-term, experimental designs.

For quantitative, routine analysis of small, repeated samples, we have adapted the gas chromatography method of Vandermark and Adams [3], which uses a very sensitive nitrogen-phosphorus detector. There are several advantages to this newly-available detector. It does not require, for small samples, the complex chemical preparation or the highly specialized glassware needed with traditional flame ionization detector (FID) gas chromatography [1,2]. While it is possible to analyze barbiturates in small (25–100 μl) blood samples with FID, the margin for error is greater and the possibility of fractionating the sample for replicated measures is limited. This paper presents gas chromatograph methodology with the nitrogen-phosphorus detector for researchers not specifically trained in this branch of biochemical analysis. Consequently, we have shown the results of varying several of the relevant parameters used in barbiturate analysis and discussed them in a way that will allow others to adapt similar procedures and validate them with their own equipment configurations and techniques.

We will present first a succinct description of the method for determining serum phenobarbital level, followed by a more complete discussion of the considerations involved in various steps of the method.

METHOD

Reagents

All reagents used are certified A.C.S. grade. (1) 1.5 M sodium phosphate monobasic. (2) 2 M TMH (0.9062 g tetramethylammonium hydroxide pentahydrate dissolved in methanol and made up to 2.5 ml). The particular TMH weight used depends on the hydration state of the TMH. In the above case, the weight is correct for the pentahydrate form. For each additional water molecule, an additional 0.09 g of the TMH would be used. (3) DMA reagent is made up with N,N-dimethylacetamide and methanol in a 1:3 ratio (v/v). (4) Cyclohexane reagent is made up with cyclohexane and methylene chloride in a 95:5 ratio (v/v). (5) Methyl iodide is certified 99.8% pure methyl iodide. (6) Ethyl acetate is certified 99.0% pure ethyl acetate. (7) Internal standard is prepared by making up a stock solution (10 mg sodium pentobarbital made up to 100 ml in methanol) and diluting each ml of this stock solution as needed to 100 ml with toluene. (8) In order to make phenobarbital standards, first a stock solution of 10 mg sodium phenobarbital made up to 10 ml with methanol is prepared. A set of phenobarbital solutions is prepared as follows:

A volume of 0.8 ml of the stock solution is made up to 10 ml with methanol in a 10-ml volumetric flask to give an 80 μg/ml solution. By a series of 1:1 volumetric dilutions with methanol, the following standards are prepared: 40, 20, 10, 5 and 2.5 μg/ml.

MATERIALS AND APPARATUS

(1) Gas chromatograph with nitrogen-phosphorus detector (Perkin-Elmer dual column chromatograph model 3920B

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with above detector). (2) Suitable chromatographic glass column (6 ft. Perkin-Elmer 3% OV-1, Gas Chrom Q, 100/120 mesh). (3) Instrument pressure regulator (similar to Perkin-Elmer 045-0188). (4) Integrator (Perkin-Elmer M-2 calculator-integrator). (5) Recorder (Perkin-Elmer 0123 recorder). (6) Automatic pipette with disposable, plastic tips. (7) Micro-syringe, 10 μl for injection into gas chromatograph (Hamilton, cat. no. 701). (8) Temperature control water bath (Precision Scientific Co., Model 81). (9) Vortex mixer (Vortex-Genie, model K-550-6). (10) Table-top centrifuge (International Equipment Co., model CL). (11) A dry airstream source. (12) Conical centrifuge tubes, 5 ml, with glass stoppers.

Apparatus Conditions

The helium flow rate through the column is set at 24 ml/min at an inlet pressure of 80 psi (5.6 kg/cm^2). The hydrogen flow rate is set at 5.6 ml/min at an inlet pressure of 20 psi (1.4 kg/cm^2). The air flow rate is set at 60 psi (4.2 kg/cm^2) at an inlet pressure of 70 psi (4.9 kg/cm^2). The gas chromatograph temperature parameters are as follows: injector 250°C, interphase 275°C, and oven 200°C. The amplifier range is set at 10 and attenuation at 8. The bead control on the nitrogen-phosphorus detector is set at 575.

Procedure

A 50 μl sample (standard or serum) is pipetted into a conical centrifuge tube containing 50 μl of 1.5 M sodium phosphate monobasic. The solution is mixed briefly (5 sec/Vortex mixer) and 2 ml of the internal standard added to the mixture. This is mixed again and centrifuged (5 min/3000 rpm). The upper layer is transported carefully to another conical centrifuge tube (disposable Pasteur pipette) which contains 50 μl of 2M TMH. This mixture is mixed well (10 sec/Vortex) and centrifuged again for 5 min. The upper layer is then discarded (all the barbiturate is concentrated in the lower alkaline layer) and 50 μl of DMA reagent is added to the lower layer. After mixing, 100 μl of methyl iodide is added. The mixture is allowed to stand for 3 min at room temperature and 1 min at 60°C (water bath). Two ml of cyclohexane reagent is then added, the mixture vortexed and centrifuged (1 min/3000 rpm). The supernatant is transferred to a conical tube and returned to the water bath where the solvent is carefully evaporated with a stream of dry air. Fifty microliters of ethyl acetate is added to the residue. The solution is mixed and the tube capped immediately. It is now ready for injection into the gas chromatograph. A 2 μl sample is used for each GC run.

RESULTS AND DISCUSSION

Figure 1 presents the chromatograms of various phenobarbital standard samples. Pentobarbital was the internal standard used. Note the clear separation between the phenobarbital and pentobarbital peaks and the relatively constant retention times between samples greatly different in concentration. In the analysis of barbital levels, it is recommended that the oven temperature be lowered to 170°C in order to achieve a better separation between the barbital and internal standard (pentobarbital) peaks.

The results of the determination can be affected greatly by changes in oven temperature, hydrogen flow rate, bead settings of the nitrogen detector and helium flow rate. These factors are quite specific to the particular system used and therefore an optimal condition should be established within

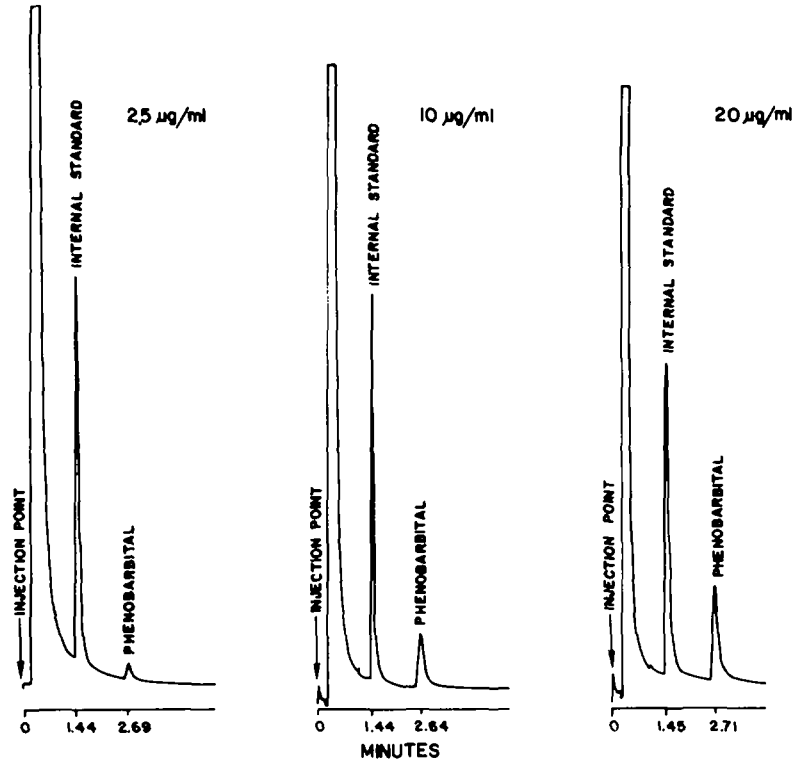

FIG. 1. Chromatograms of 3 phenobarbital standard samples. The internal standard used was pentobarbital.

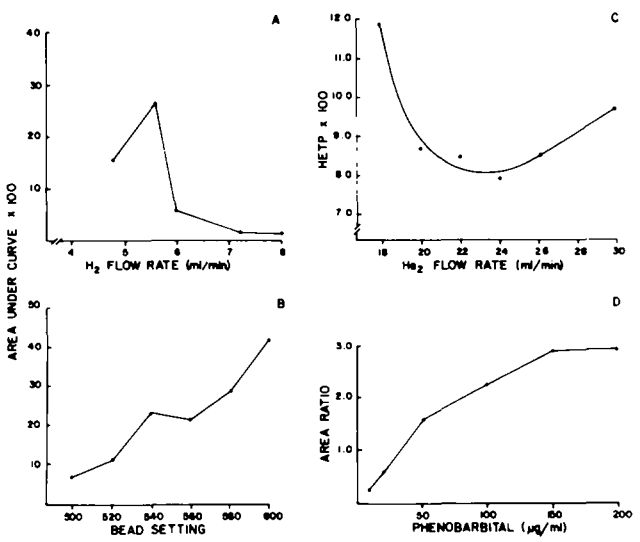

FIG. 2. Panels A and B show the effects of hydrogen flow rate and bead setting on detector sensitivity. The functional relation between helium flow rate and HETP values is presented in Panel C. Panel D presents the calibration curve for phenobarbital concentrations ranging from 5 to 200 µg/ml.

each laboratory. The following paragraphs discuss various considerations that are important in the choice of the final parameters. The effects of these variables on the determination will be examined in a control situation in which the level of each variable was changed systematically while holding all other factors constant.

Oven Temperature

The amount of time needed for each analysis depends upon the temperature of the oven: the higher the temperature, the shorter the time. However, if the oven temperature is set too high the various peaks arrive too closely in time and consequently any resolution between them is difficult. On the other hand, if the temperature is set too low, the time needed for each analysis is too long. In addition, the peaks would be flattened and also there would be a tendency for tailing to occur.

Hydrogen Flow Rate

The sensitivity and selectivity of detection can vary as much as 16 times with a difference of less than 3 ml/min in hydrogen flow rate (see Panel A, Fig. 2). During initial determinations, it is safer to start with lower settings and slowly move higher. Too rapid an input of hydrogen could result in the burning up of the nitrogen to be detected, resulting in failure of the detector to pick up any signal at all.

Bead Setting

The sensitivity of the detector was found to be a linear function of increasing detector bead setting (Panel B, Fig. 2). Unfortunately, the life of the bead is also a linear function of bead setting.

Helium Flow Rate

It is recommended that a low rate of helium (10 ml/min with 20 psi at the inlet) be allowed to flow through the column at all times (when not analyzing samples) in order to prevent contamination of column packing material by atmospheric air. When changing flow rates, at least 5 min is needed for the flow rate to stabilize at the new level. During an analysis, the optimal helium flow rate is the one that yields the smallest HETP (height equivalent to theoretical plate) value. $HETP=L/16(x^2/y)$ when L=length of column used; x=time between injection of sample into the gas chromatograph and the arrival of the peak maximum of the compound under study; y=base width of that peak. With our system, 24 ml/min was found to be the optimal rate for helium flow (Panel C).

Sample Concentration

Panel D presents the calibration curve for phenobarbital obtained under conditions detailed in the method section. Area ratio was calculated by dividing the area of phenobarbital by the area of internal standard with the aid of the Perkin-Elmer M-2 integrator/calculator. Using this method, linear results were obtained with samples in the range of 5–50 µg/ml. Differentiation between 150 and 200 µg/ml was not possible. Thus, we have routinely diluted samples that yielded values greater than 50 µg/ml. We have recently upgraded our Model M-2 to a Perkins-Elmer Sigma 10 Chromatograph Data Station which yields linearity up to a phenobarbital concentration of 100 µg/ml.

Reliability of the method may be checked by the size of the coefficient of variation (CV%). Table 1 shows the data of 6 replicates at each of 4 phenobarbital concentrations. Acceptable CV% is a function of the number of samples used and the discriminative precision required by the particular experiment.

TABLE 1

AREA-RATIOS OF 6 REPLICATE ANALYSIS AT EACH OF 4 PHENOBARBITAL CONCENTRATIONS

Samples	Phenobarbital Concentrations			
	5 µg/ml	10 µg/ml	20 µg/ml	40 µg/ml
1	0.1463	0.3773	0.6523	1.6239
2	0.1624	0.3454	0.6315	1.5879
3	0.1704	0.3594	0.6549	1.4879
4	0.1573	0.3567	0.6429	1.7466
5	0.1566	0.2853	0.7315	1.3974
6	0.1653	0.3098	0.6676	1.4903
Mean	0.1597	0.3390	0.6635	1.5557
S.D.	0.008	0.034	0.036	0.1232
CV%*	5.009	10.03	5.43	7.92

* $CV\% = \frac{S.D. \times 100}{MEAN}$

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