Journal of Chromatography, 229 (1982) 365—372
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1213

CHROMATOGRAPHIC MICRO-PROCEDURE FOR TRACE DETERMINATION OF PHENOBARBITAL IN BLOOD SERUM

A. DI CORCIA*, L. RIPANI and R. SAMPERI

Istituto di Chimica Analitica, Università di Roma, 00185 Rome (Italy)

(First received October 6th, 1981; revised manuscript received December 29th, 1981)

SUMMARY

Volumes of 100 μl of serum were sufficient for the determination of therapeutic levels of phenobarbital. The isolation procedure was performed using a column method with a hydrophobic adsorbent, graphitized carbon black (Carbopack B). With this method the quantitative (98.1%) recovery of phenobarbital was measured. By suitable choice of experimental conditions, a highly selective purification of the drug can be obtained, thus eliminating various sources of error during quantitation due to the presence in the final samples of endogenous compounds. For the quantitation procedure, another type of graphitized carbon black (Carbopack C) suitably modified was used for gas chromatography. Calibration curves showed no chemisorption effect along the column even on injecting 5 ng of phenobarbital. Some practical aspects of the procedure for improving the reliability of the results are discussed.

INTRODUCTION

Determination of the blood concentrations of barbiturates, and in particular of phenobarbital, is of importance in the treatment of epilepsy and in the diagnosis of acute poisoning. For this purpose, gas chromatographic (GC) and, more recently, high-performance liquid chromatographic (HPLC) procedures have been a great aid in clinical laboratories since they meet the requirements for rapid identification and accurate quantitation.

Many of the GC methods currently available succeed in the analysis of drug overdoses, but they fail in the case of therapeutic monitoring, where the blood drug concentration is much lower. Chemisorption of acidic drugs occurring along the chromatographic column and/or in the injection port is often responsible for this failure. Recently, it has been reported [1] that a typical chromatographic column used for drug analysis, i.e. phenylsilicone supported by an H_3PO_4-deactivated siliceous material, is inadequate to determine

0378-4347/82/0000—0000/$02.75

accurately phenobarbital concentrations lower than 15 μg/ml, due to partial irreversible adsorption of phenobarbital on the column.

Chemisorption occurring on the injection port is likely caused by insufficient isolation of drugs from endogenous biological molecules, such as peptides, proteins, nucleotides, etc. These molecules are denatured on the injection port and can act as centers of chemical adsorption of acidic compounds [2].

This drawback can be eliminated by derivatization procedures prior to GC [3—5] and/or by clean-up procedures following extraction of drugs from biological fluids [6, 7]. These additional steps in the analytical scheme are time-consuming, however, and can provoke loss of the sample due to excessive manipulation.

HPLC has proved to be valid for the analysis of drugs and their metabolites in biological fluids [8, 9]. Nevertheless, even in this case a complete isolation of drugs from matrix material in clinical samples is necessary to avoid degradation of the column to a point where the system becomes unusable.

Graphitized carbon black (Carbopack) has been successfully used as packing material in GC of very polar compounds [10—13]. Recently, this adsorbing material has shown to be effective in the purification and concentration of pesticides and other organic molecules in water [14, 15].

In this paper, an accurate and simple micro-method for the determination of phenobarbital in serum at a few μg/ml is described. A small (100 μl) volume of the biological specimen was purified using a column filled with Carbopack B. Phenobarbital was desorbed with methanol as mobile phase. Analytical recoveries ranged from 96.1 to 99.7% for phenobarbital in serum. The quantitation procedure was performed using a GC column packed with acid-washed Carbopack C modified with Apiezon and polyethylene glycol (PEG) 20M. The advantages of using the adsorption method over solvent extraction for the purification of acidic compounds in biological samples are demonstrated.

MATERIALS AND METHODS

Chemicals

Phenobarbital, cyclohexenylbarbital and cycloheptenylbarbital were obtained from Fluka (Buchs, Switzerland). Carbopack B and C in the 80—120 mesh range were kindly supplied by Supelco (Bellefonte, PA, U.S.A.). PEG 20M and Apiezon N were purchased from Carlo Erba (Milan, Italy). 1,3,5-Tricarboxybenzene (trimesic acid) was obtained from Fluka.

Gas chromatography

A Carlo Erba Model GI gas chromatograph equipped with a flame ionization detector was employed. Integration of peak areas was performed by a Hewlett-Packard 3352 data system. The column, a coiled glass tube (1 m × 1.5 mm I.D.), was washed with trimesic acid in methanol (ca. 1 mg/ml) in order to deactivate basic centers of glass and dried under vacuum. Subsequently, it was packed with acid-washed Carbopack C modified with PEG 20M (0.3%, w/w), Apiezon N (0.28%, w/w) and trimesic acid (0.1%, w/w). This latter is added to increase the thermal stability of PEG 20M. Trimesic acid was deposited on the Carbopack C surface from methanol, PEG 20M and Apiezon N were deposited

from methylene chloride. The procedures of acid-washing, coating and packing have been reported elsewhere [16, 17]. No glass wool was used to plug the injection end of the column. The column was conditioned at 250°C overnight under flow.

For the quantitation procedure, the column was operated at 235°C with hydrogen as carrier gas, which offers advantages over nitrogen [18]. With a dead-time of 6 sec, phenobarbital was eluted within 12 min. The retention time was longer (16 min) by operating with nitrogen (dead-time 10 sec) at 240°C. Under the former experimental conditions, the chromatographic column was perfectly stable. A 3% variation of the retention time for phenobarbital was measured after 72 h of use at 240°C. This variation did not modify the separation factors of barbiturates and it was minimized by maintaining the column temperature at 150°C when not in use. With this precaution, the column could be used continuously for three months.

During chromatographic analysis, the injection port temperature was maintained at 250°C to ensure instantaneous vaporization of barbiturates. At lower temperatures, a certain peak broadening for these compounds was noted.

Procedure

The column for isolating phenobarbital from serum was prepared by suspending 0.6 g of Carbopack B in water and pouring the slurry into a 14 × 0.6 cm glass column with a PTFE stopcock. The adsorbent was packed by tapping the column while water passed through it. This operation was stopped when the level of carbon reached a height of 6 cm. The dead volume of the column was 1.2 ml; this was calculated by passing 1 *M* HCl through the column and measuring the acidity of the effluent. A 0.1-ml volume of serum containing phenobarbital was added to the head of the column and allowed to drain into the column. When the level of serum reached that of the adsorbent, 4 ml of 1 *M* HCl in water were passed through the column to denature proteins which are eliminated from the column as precipitate. After the acidic solution was passed into the column, the adsorbent was washed with 1.5 ml of a methanol—water (1:1) mixture to eliminate the major part of the acidic water. Methanol was used to desorb phenobarbital from the carbon surface. During elution, the flow-rate was maintained at 0.5—0.7 ml/min. The first 6.8 ml of the effluent of the column were discarded and the following 1 ml containing phenobarbital was collected. The discarded fraction consists of 1.2 ml of dead volume, 0.1 ml of serum and 5.5 ml of washings. Then 10 μl of a methanolic solution of cycloheptenylbarbital (0.05 mg/ml), used as internal standard, were added to the collected fraction. After this, the fraction was submitted to a stream of nitrogen at room temperature to remove methanol completely from co-eluted water. A final volume sample of about 6—8 μl was obtained. This sample was then analyzed by injecting aliquots of 1.5 μl into the GC column.

RESULTS AND DISCUSSION

Column

In order to ascertain whether some chemisorption of acidic drugs can occur in the GC apparatus, calibrated test solutions containing phenobarbital

TABLE I

RESPONSE FACTOR FOR PHENOBARBITAL vs. OCTADECANE AT VARIOUS CONCENTRATIONS

Concentration (μg/ml)	Mean ± S.D. (n = 6)
5.00	0.517 ± 0.030
12.5	0.509 ± 0.029
25.0	0.491 ± 0.018
50.0	0.493 ± 0.021
100.0	0.507 ± 0.018

dissolved in chloroform at concentrations ranging from 5 to 100 μg/ml, and octadecane, as internal standard, were prepared. For each solution, 1-μl aliquots were repeatedly injected into the column. Partial chemisorption of a given compound by the chromatographic column can be adequately recognized by injecting variable amounts of the compound examined together with an inert eluate and measuring changes of the response factor at any given concentration. Results are shown in Table I. As can be seen, by injecting phenobarbital solutions at increasing concentrations no regular increase of the response factor was observed. This demonstrates that even by injecting a few nanograms of phenobarbital into the column a complete, linear elution is obtained. Also, a well-defined peak for phenobarbital, whose area could be measured with an uncertainty not higher than 4%, was obtained by injecting 5 ng of phenobarbital (Fig. 1).

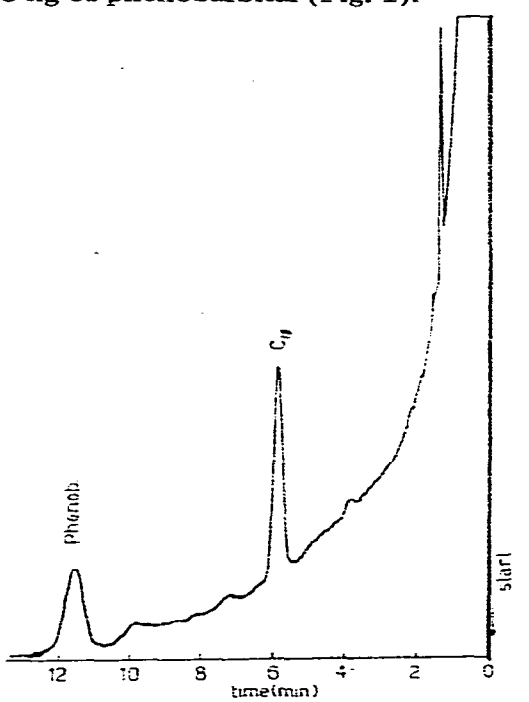

Fig. 1. Chromatogram of a methanol solution containing phenobarbital (5 μg/ml) and C_{18} (2.5 μg/ml) at 235°C and with hydrogen as carrier gas.

Even after injecting a large number of biological samples, the column packing did not show any need to be deactivated or restored or substituted. This is also because liquid-modified Carbopack C was very tolerant to injections of the aqueous sample containing drugs. The advantage of injecting water over organic solvents is that a much smaller baseline drift is obtained.

Recovery

Recovery studies were performed by adding specified quantities of phenobarbital to pooled sera known to be drug-free and contained in glass tubes with PTFE-lined screw caps. Then 0.1-ml sample aliquots were submitted to the purification procedure and 10 μl of the methanolic solution of cycloheptenylbarbital (0.05 mg/ml), used as internal standard, were added to the purified sample. After concentration, replicate injections of the sample were made. The peak areas of the extracted drug relative to those of the internal standard were compared with the relative peak areas obtained by injecting standard solutions prepared by adding to water known amounts of phenobarbital and internal standard. Recoveries using the adsorption column method on six determinations varied between 96.1 and 99.7% with a mean of 98.1 ± 1.1% for concentrations of phenobarbital in serum ranging from 4 to 20 μg/ml.

Specificity

The analytical procedure discussed here can be used for the determination of barbiturates other than phenobarbital in blood. The analytical column was only slightly modified with respect to one used previously, which was able to separate the most commonly used barbiturates [19].

Under the experimental conditions chosen for the isolation procedure, no interference from endogenous sample constituents was observed. This was made evident by the fact that on analyzing twenty drug-free human serum samples no measurable peaks with retention times equal to those of barbiturates appeared. Fatty acids and cholesterol contained in serum are not co-eluted from the adsorption column, provided methanol is used as the mobile phase. Selective elution from the column filled with Carbopack B is lost when methylene chloride and, to a lesser extent, acetone are used as mobile phases. In this case, fatty acids contained in blood interfere with some barbiturates in the quantitation procedure when using our GC column. Cholesterol did not interfere in the analysis because its retention time is far higher than that of cycloheptenylbarbital, which is the last of the barbiturates to be eluted. However, the presence of cholesterol did reduce the rate at which these analyses could be performed.

Limits of sensitivity

It has been shown [20] that the limit of sensitivity towards phenobarbital is influenced by chemisorption which may occur on-column. As mentioned above, this problem does not occur when using acid-washed Carbopack C suitably modified. The chromatographic system permitted us to measure accurately peaks for phenobarbital contained in standard aqueous solutions at the 5 μg/ml level. Considering that after the isolation and concentration procedures the final sample volume is about 6—8 μl (note that this sample volume

allows replicate 1.5-μl injections), it can be deduced that blood phenobarbital concentrations of about 0.5 μg/ml can be still measured with fair accuracy. Alternatively, considering that phenobarbital concentrations achieved during therapy are usually in the range 3—8 μg/ml, very small (10—20 μl) serum volumes may be used without affecting the quality of the analysis. An example of the chromatogram normally obtained on analysis of phenobarbital in human serum (100 μl) at the 5 μg/ml level is given in Fig. 2.

Comparison with the results obtained using the extraction method

The isolation method under discussion was evaluated by comparison with results from the solvent extraction method. An extraction procedure similar to that commonly reported in the literature was followed. To 1 ml of acidified serum 10 μl of a methanol solution of phenobarbital and cylcohexenylbarbital, used as internal standard, (1 mg/ml each) were added. Then, 2 ml of chloroform were used for extraction by shaking for 2 min and centrifuging for 10 min. Subsequently, a 1.5-ml portion of the chloroform phase was obtained with a syringe fitted with a 9-cm needle, taking care not to withdraw denatured proteins present at the interphase. After this, the organic solvent was evaporated with a stream of nitrogen at 40°C. For the GC analysis, the extract was reconstituted with about 20 μl of chloroform and 2-μl aliquots of the extract were injected ten times into the GC apparatus followed by 1 μl of 0.1 *M* formic acid in water. As can be seen in Fig. 3a, by injecting acidified water two "ghost" peaks having retention times equal to those of phenobarbital and cylcohexenylbarbital appeared. This effect can be explained by the

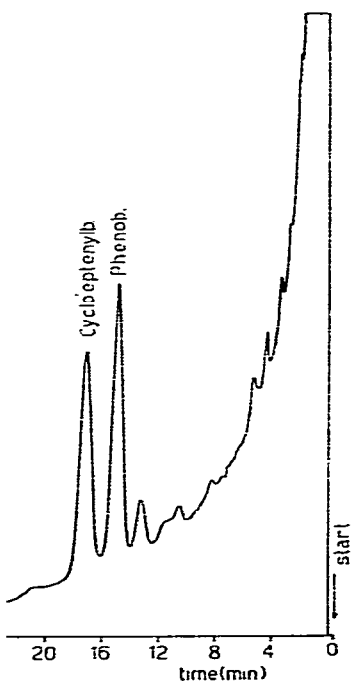

Fig. 2. Analysis of purified serum (100 μl); 1.5 μl injection. The serum concentration of phenobarbital was 5 μg/ml.

consideration that endogenous substances co-extracted with the two drugs are thermally denatured and accumulate in the injection port, provoking chemisorption of acidic compounds. Acidic drugs can be removed from the initial part of the column by injections of a stronger acidic compound. Selective chemisorption towards the compounds injected can give rise to erratic results to a greater or lesser extent depending upon the nature of the compound chosen as internal standard. It can be also expected that a prolonged number of analyses increases the degree of contamination of the injection port, thus increasing the effect of chemisorption to a point where the chromatographic system is unusable.

By using solvent extraction for isolating barbiturates and the GC system discussed here, another limiting factor is that co-extracted fatty acids are interfering compounds for the quantitation and identification of some barbiturates.

In Fig. 3b is shown a chromatogram obtained by injecting formic acid in water just after 40 injections of samples purified with the adsorbent column method following the procedure described above. The complete absence of "ghost" peaks makes it evident that this purification method is effective in isolating drugs from endogenous compounds. It should be pointed out, however, that for any class of compounds the column method using Carbopack necessitates a careful choice of the procedure to be followed in order to obtain the maximum degree of purification of biological samples. In our instance, methylene chloride was not found suitable as mobile phase since barbiturates are co-eluted with fatty acids. Moreover, washing the column with acidified water before using methanol was found necessary to remove from the column substances that are eluted with methanol and are responsible for chemisorption of acidic compounds on the injection port.

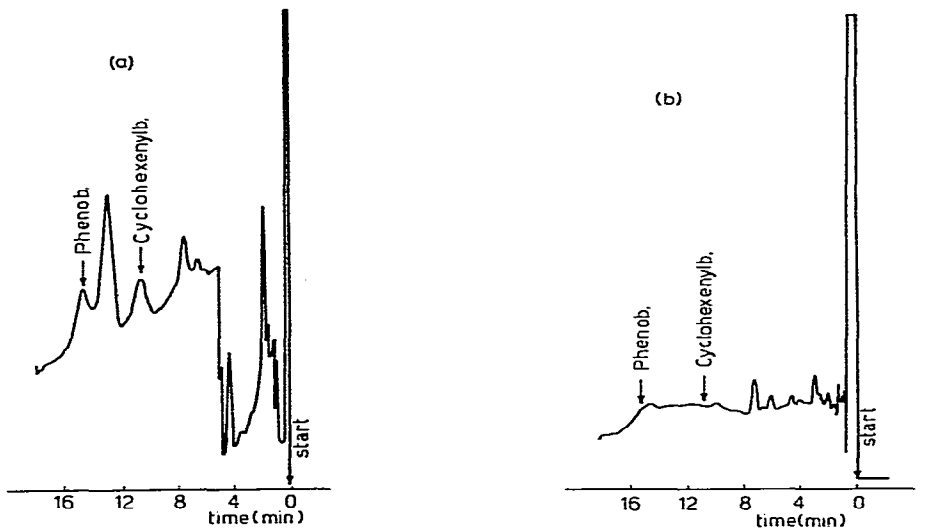

Fig. 3. (a) Chromatogram showing the appearance of two "ghost peaks" for injection of acidic water following injections of an extract of serum. (b) No ghost peaks for injection of acidic water following injections of serum purified by using Carbopack B.

CONCLUSION

The procedure described here shows the advantages of minimal sample, high sensitivity, good accuracy and reproducibility for analyzing trace concentrations of phenobarbital in serum. The isolation method proposed by us can be very selective, thus eliminating interferences from endogenous compounds. Another virtue of this method is its flexibility, since any kind of solvent or mixtures of these can be used to elute selectively the compounds of interest.

ACKNOWLEDGEMENTS

The authors want to thank Mr Gianni Cosmi for his excellent experimental assistance and Mrs. Doralba Berardo for typing the manuscript.

REFERENCES

1 L.F. Jaramillo and J.N. Driscoll, J. Chromatogr., 114 (1979) 637.
2 B. Pileire, J. Chromatogr., 162 (1979) 446.
3 H. Roseboom and A. Hulshoff, J. Chromatogr., 717 (1979) 66.
4 E. Hill and A.N. Latham, J. Chromatogr., 131 (1977) 341.
5 F.L. Vandemark and R.F. Adams, Clin. Chem., 22 (1976) 1062.
6 R.F. Adams and F.L. Vandemark, Clin. Chem., 22 (1976) 25.
7 A. Toseland, J. Grove and D.J. Berry, Clin. Chim. Acta, 38 (1972) 321.
8 R.F. Adams, G.J. Schmidt and F.L. Vandemark, J. Chromatogr., 145 (1978) 275.
9 D.J. Freeman and N. Rawal, Clin. Chem., 25 (1979) 810.
10 A. Di Corcia, Anal. Chem., 45 (1973) 492.
11 A. Di Corcia and R. Samperi, Anal. Chem., 46 (1974) 140.
12 A. Di Corcia and R. Samperi, Anal. Chem., 46 (1974) 977.
13 A. Di Corcia and R. Samperi, Anal. Chem., 51 (1979) 776.
14 A. Bacaloni, G. Goretti, A. Laganà, B.M. Petronio and M. Rotatori, Anal. Chem., 52 (1980) 2033.
15 F. Mangani, G.Crescentini and F. Bruner, Anal. Chem., 53 (1981) 1627.
16 A. Di Corcia, R. Samperi, E. Sebastiani and C. Severini, Anal. Chem., 52 (1980) 1345.
17 A. Di Corcia, A. Liberti and R. Samperi, J. Chromatogr., 122 (1976) 459.
18 A. Di Corcia and M. Giabbai, Anal. Chem., 50 (1978) 1000.
19 A. Di Corcia, A. Liberti and C. Severini, J. Chromatogr., 204 (1981) 255.
20 D.M. Rutherford and R.J. Flanagan, J. Chromatogr., 11 (1978) 311.