Gas-Chromatographic Determination of Mephenytoin and Desmethylmephenytoin, after Off-Column Alkylation

Vidmantas A. Ralsys, Arthur M. Zebelman,¹ and Stuart F. MacMillan

We describe a gas–liquid chromatographic method for determining mephenytoin and its active metabolite, desmethylmephenytoin, in human serum. 5-Methyl-5-phenylhydantoin, is used as the internal standard. The method involves extraction of the drugs by adsorption onto charcoal and off-column derivatization to their penty1 derivatives. Peak height and concentration are linearly related and the day-to-day CV for therapeutic concentration is about 2 to 6%. No interferences by endogenous compounds or drugs commonly used for seizure control have been encountered.

Mephenytoin (3-methyl-5-ethyl-5-phenylhydantoin) was first introduced in 1945 as an anticonvulsant drug (1). Early studies showed that mephenytoin was effective in control of grand mal and focal seizures (2-5). Although mephenytoin causes fewer side effects such as nausea, ataxia, and gum hyperplasia than does phenytoin, the incidence of serious dermatitis, agranulocytosis, anemias, and hepatitis is greater in patients being treated with mephenytoin for seizure control (6). Fatal cases of aplastic anemia have been also reported (6, 7). In spite of these side effects, Troupin et al. (7) recommend a wider use of this drug by patients in whom seizures are difficult to control.

Mephenytoin is metabolized by demethylation to desmethylmephenytoin (5-ethyl-5-phenylhydantoin), an active metabolite (8). To correlate clinical effects and drug concentration in serum, one must measure both mephenytoin and desmethylmephenytoin (7). The therapeutic concentration for the two together reportedly is 15-40 mg/L (9).

The fact that therapy with mephenytoin has been associated with relatively high incidences of serious side effects has led to its use mainly in patients with severe grand mal seizures which are resistant to less toxic agents, and has motivated us to develop an assay for mephenytoin and desmethylmephenytoin that is appropriate for routine use in the clinical toxicology laboratory.

Several gas-chromatographic methods have been reported for mephenytoin and desmethylmephenytoin determination (10-12). The method of Kupferberg and Yanekawa (11) is long and involves double extraction. The method of Friel and Troupin (12) is based on a modification of the MacGee method for on-column alkylation (13). In our hands, and as noted by Friel and Troupin (12), the on-column ethylation procedure is sensitive to various manipulative factors in the procedure. Off-column alkylation procedures for several drugs have been reported (14, 15), and our method is based on these observations. The method involves extraction of mephenytoin and desmethylmephenytoin with charcoal and off-column derivatization of the drugs followed by gas-chromatographic determination.

Materials and Methods

Apparatus

We used a dual column Hewlett-Packard Model 5700 gas-liquid chromatograph equipped with dual-flame ionization detectors (Hewlett-Packard, Palo Alto, CA 94303). A glass column (183 cm × 2 mm i.d.) packed with 3% OV-225 on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, PA 16801) was found most suitable. The following gas-chromatographic settings were used: column temperature, 230 °C isothermal; detector temperature, 250 °C; injection temperature 230 °C; helium flow-rate, 45 mL/min; air flow-rate, 270 mL/min; and hydrogen flow-rate, 30 mL/min.

Reagents

Pure reference samples of mephenytoin and desmethylmephenytoin were obtained from Sandoz Pharmaceuticals, East Hanover, NJ 07946. The internal standard, 5-methyl-5-phenylhydantoin, was purchased from Aldrich Chemical Co., Milwaukee, WI 53233. Stock solutions of the drugs were prepared in absolute methanol in 1 g/L concentration and stored at −20 °C. Working standards, mephenytoin 5–20 mg/L, and desmethylmephenytoin 10–40 mg/L were prepared in a 50 g/L solution of bovine serum albumin, preserved with sodium azide (1 g/L) and stored at −20 °C.

Phosphate buffer (0.25 mol/L, pH 6.0) was prepared from monobasic potassium phosphate and the pH adjusted with 2 mol/L sodium hydroxide solution. The extraction buffer was prepared by adding 2 mL of the stock 5-methyl-5-phenylhydantoin to 98 mL of phosphate buffer and stored at 4 °C.

Trimethylphynylammonium hydroxide (250 g/L, in methanol) was prepared from trimethylphenylaminilium iodide (Eastman Kodak Co., Rochester, NY 14650) by the method of Skinner et al. (16). Just before use, 100 μL of the methanolic trimethylphenylammonium hydroxide was evaporated under an air stream and reconstituted with 1 mL of N,N-dimethylacetamide (Aldrich Chemical Co.).

A mixture of penty1 iodide (Eastman Kodak Co.) and N,N-dimethylacetamide (1/10 by vol) was prepared just before use.

Norit A neutral charcoal was purchased from Amend Drug and Chemical Co., Irvington, NJ 07111.

All solvents used were analytical grade or better.
Procedure

Add 1 mL of serum or standard, 1 mL of extraction buffer, and about 20 mg of charcoal to 15 × 125 mm culture tube with a poly(tetrafluoroethylene)-lined cap. (The amount of charcoal added to each tube was visually estimated after an initial weighing.) Vortex-mix the mixture for 15 s and then centrifuge for 5 min at 1000 × g. Aspirate and discard the supernate. To the charcoal, add 2 mL of dichloromethane and vortex-mix for 30 s. Decant the dichloromethane into a clean 15-mL glass centrifuge tube and evaporate it in a stream of air, with the tube in a water bath at 55 °C. To the residue add 50 μL of pentyl iodide in N,N-dimethylacetamide and 25 μL of trimethylphenylammonium hydroxide in N,N-dimethylacetamide. Vortex-mix for 5 s and allow to react for 10 min at room temperature. Then add 0.5 mL of cyclohexane/dichloromethane (95/5 by vol) solution to precipitate the excess of derivatizing reagents. Centrifuge for 5 min at 1000 × g. Decant the supernate into a clean 13 × 100 mm culture tube and evaporate as before. Dissolve the residue in 50 μL of methanol and inject 1 μL into the gas chromatograph.

Results and Discussion

Figure 1 depicts some representative chromatograms of serum taken through our method. The separation of caffeine from mephenytoin is incomplete but adequate, as shown in Figure 1C. Table 1 lists absolute and relative retention times of drugs most frequently prescribed for patients undergoing anticonvulsant therapy. None of these drugs interferes in the present method.

Quantitation of mephenytoin and desmethylmephenytoin is based on the relative peak height ratios with 5-methyl-5-phenylhydantoin as the internal standard. Figure 2 shows a typical standard curve for mephenytoin and desmethylmephenytoin. Mephenytoin standards in the range of 5–20 mg/L and desmethylmephenytoin 10–40 mg/L are used in the assay. Since the concentrations of mephenytoin in the serum of patients receiving therapy with mephenytoin averages about 8% of the total active drug, mephenytoin plus desmethylmephenytoin (7), we have elected to use lower concentrations of mephenytoin in our standards. The curves for both mephenytoin and desmethylmephenytoin are linear, as is shown in Figure 2.

Within-run precision of the assay was evaluated from data
on 10 replicate determinations of the drugs prepared in pooled serum (Table 2). Table 2 also summarizes between-day precision studies, with data from 20 different determinations of mephenytoin and desmethylmephenytoin at two concentrations each.

We evaluated the accuracy of the method by a recovery study. Drug-free serum was supplemented with mephenytoin and desmethylmephenytoin at two different drug concentrations and the recovery calculated (Table 3).

We examined the correlation between our method and the method of Friel and Troupin (12). These authors kindly supplied us with 10 patients' samples that they had already analyzed for mephenytoin and desmethylmephenytoin according to their published procedure (12). Table 4 shows the correlation of our data with theirs for mephenytoin, desmethylmephenytoin, and the total of both drugs. Figure 3 shows the correlation for total drug concentration. The correlation coefficients are all quite satisfactory, but there is a difference for mephenytoin concentration. This may be related to the very low mephenytoin concentration in the samples that we analyzed; the mean mephenytoin concentration for these 10 samples was 2.4 mg/L. We have measured the recovery by our method in this range and found it to be 97% (Table 3), which we consider to be satisfactory. We have also assayed standard samples supplied by Friel and Troupin, with excellent results. We cannot explain the discrepancy between the two methods that we have observed for low mephenytoin concentrations.

The method that we have presented can be used in routine clinical chemistry laboratory determinations for mephenytoin and desmethylmephenytoin. It is accurate and precise. Using off-column alkylation extends the column life by avoiding injections of deleterious alkylation reagents onto the column. This method has been used in our laboratory for over a year, on a weekly schedule, with no major problems.

This work was supported in part by NRSA Research Fellowship 1F32HD05381-01 and by NIGMS Training Grant in Clinical Chemistry 5T01GM00776 from the NIH, Bethesda, MD.

References
8. Butler, T. C., Metabolic demethylation of 3-methyl-5-ethyl-5-

C L I N. C H E M. 25/1, 175-178 (1979)

Performance of the Du Pont aca Ammonia Method

Basil T. Doumas,1 Lawrence L. Hause,1 Richard D. Sciacca,1 Bernadine Jendrzejczak,1 Craig C. Foreback,2 John D. Hoover,2 W. William Spencer,3 and Paul L. Smock3

We evaluated the performance of the Du Pont aca ammonia procedure with regard to (a) linearity, (b) precision, (c) interferences, and (d) effect of anticoagulants. Linearity extends to 2000 μmol/L. The SD of the method was essentially constant (2 to 3 μmol/L) and independent of the NH3 concentration. Hemoglobin, bilirubin, and lipemia do not interfere. Either EDTA or heparin is suitable as anticoagulant. Recovery of NH3 added to plasma samples averaged 102% (range: 97–107%). We established normal values by measuring NH3 in 188 plasma samples from apparently healthy individuals. The 95% confidence range is from 10 to 35 μmol/L. The aca ammonia method compares very well with that of Kingsley and Tager but correlates less strongly with that of Reinhold and Chung. We describe a protein-based solution with stable NH3 concentration that is suitable as a control material.

Additional Keyphrases: variation, source of normal values · intermethod comparison

An evaluation of the Du Pont aca procedure has been published recently by JPMat et al. (1). The same procedure has been evaluated by our three hospital laboratories. Our studies have been considerably more extensive and include a determination of normal range, effect of anticoagulants, stability of ammonia in blood samples, preparation of a suit-

1 Department of Pathology, The Medical College of Wisconsin, and The Milwaukee County Medical Complex, 8700 W. Wisconsin Ave., Milwaukee, WI 53226.
2 Henry Ford Hospital, Detroit, MI 48202.
3 St. Elizabeth Medical Center, Dayton, OH 45469.
Received Aug. 25, 1978; accepted Nov. 3, 1978.

13175