Antiepileptic Agents—Primidone, Phenobarbital, Phenytoin, and Carbamazepine by Reversed-Phase Liquid Chromatography

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Introduction

Epilepsy occurs in as much as 2% of the North American population and may be found in every community. Proper diagnosis and appropriate drug therapy may keep 75% of people with epilepsy symptom-free. Some of the other 25% may respond to an alternative form of therapy or have some symptoms relieved by specific drug therapy.

Convolusions are the result of a variety of causes, not all of which are epilepsy. In addition, not all epileptic seizures are convulsions. Thus, in the context of the analyses discussed here, the term antiepileptic agents is more appropriate than anticonvulsant.

There are several classifications of epilepsy. Use of a uniform classification allows a more nearly accurate evaluation of drug effects. Investigators have been able to show that certain antiepileptic agents are more or less useful in different clinical settings (1). Modern antiepileptic agents can be divided into several groups. Which kind to use is a complex topic on which there is not universal agreement. Frequently, these agents are used together in various combinations.

Monitoring is desirable for several reasons: (a) patient compliance may be documented; (b) dosage may be adjusted to drug concentrations, the differences in absorption, metabolism, and excretion among patients making dosage adjustments difficult otherwise; (c) for several antiepileptic agents, clinical response has been demonstrated to be related to total circulating drug concentration; (d) toxic concentrations, which have been identified for most antiepileptic agents, may be avoided (2).

Principle

The antiepileptic agents primidone, phenobarbital, phenytoin, and carbamazepine are extracted into a chloroform/methanol solvent mixture. An aliquot of the extract is evaporated, reconstituted with mobile phase, and injected. The drugs are separated by reversed-phase “high-pressure” liquid chromatography and emerge in the order stated (Figure 1). The effluent is monitored at 195 nm and 254 nm. Quantification is by the peak-height ratio method.

Materials and Methods

Specimen Collection and Handling

Type: Serum (see Note 4, preceding Results and Discussion).

Storage: 4–8 °C until analysis, not to exceed 24 h.

Reagents

Filter all solvents before use. Use Teflon 0.45-µm (pore size) filters for organic solvents and cellulose ester 0.45-µm filters for aqueous solvents (See Apparatus, no. 14).

1. Acetonitrile, chromatography grade (cat. no. 0150; Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442).
2. Methanol, chromatography grade (cat. no. MX483; MCB Manufacturing Chemists, Cincinnati, OH 45212).
3. Potassium phosphate, monobasic (1 mol/L). Place 13.6 g of KH₂PO₄ (certified ACS, cat. no. P285; Fisher Scientific Co., Pittsburgh, PA 15219) in a 100-mL volumetric flask and bring to volume with de-ionized water. This is stable for six months in a dark container at 4–7 °C.
4. Phosphoric acid (0.9 mol/L). Place 10.4 mL of 85% phosphoric acid (certified ACS, Fisher cat. no. A242) in a 100-mL volumetric flask and bring to volume with de-ionized water. This is stable for at least six months in a dark container at 4 °C.
5. Sodium hydroxide, 500 g/L (cat. no. 1-3727; J. T. Baker Chemical Co., Phillipsburg, NJ 08865).
6. Phosphate buffer (pH 4.4). Add 0.60 mL of 1 mol/L KH₂PO₄ solution to 4 L of de-ionized water, followed by 0.2 mL of 0.9 mol/L phosphoric acid. Adjust to pH 4.4 with 0.1 mol/L NaOH or 0.9 mol/L phosphoric acid. Filter before use (HAWP 040700 filter; Millipore Corp., Bedford, MA 01730). This is stable for at least two days at 20 °C.
7. Mobile phase. Mix 1300 mL of filtered phosphate buffer (pH 4.4), 420 mL of filtered methanol, and 280 mL of filtered acetonitrile, then degas. This is stable for at least two weeks at 20 °C.
8. De-ionized water.
10. Ammonium sulfate solution (100 g/L). Add 20 g of
used a solvent delivery system (Model 6000A), universal liquid-chromatograph injector (Model U6K), absorbance detector with 254-nm filter (Model 440), and a variable-wavelength detector, set at 195 nm (Model 450), all from Waters Associates, Milford, MA 01757.

2. Reversed-phase column (μBondapak C_{18}; Waters Associates), 250 × 4.6 mm (i.d.).

Note: Evaluator L.A.B. reports that a Spherisorb ODS (10-μm particle size) column may be substituted for the μ-Bondapak C_{18} column; the order of elution, the limits of linearity, and the recovery are the same.

3. Dual-pen recorder (Houston Instruments, Omniscribe Model B5217-1, available from Waters Associates).
4. Magnetic stirrer (Model PC353; Corning Medical, Medfield, MA 02502).
5. Centrifuge (Dynac II, 2500 rpm; Clay Adams, Parsippany, NJ 07054).
6. Pipettes: 50-, 100-, and 250-μL Eppendorf pipettes (Fisher cat. nos. 21-370E, 21-370G; and 21-370J); 25-μL Hamilton pipette (Waters Associates cat. no. 84816); 14.8-cm (length) transfer pipette (cat. no. 063-156; Curtin-Mathes-son Scientific, Houston, TX 77001); 10-mL serological pipette.
7. Glass tubes: 16 × 100 mm, 12 × 75 mm.
8. Drying manifold, supplied with air.
10. Timer.
11. Precision pipette tips (Lancer).
12. Vortex-type mixer.
14. Flexible laboratory film (Parafilm M; American Can Co., Greenwich, CT 06830).
15. Water bath at 55 °C.

Procedure

1. Set chromatographic conditions as follows:
   - Column: μ-Bondapak C_{18}
   - Temperature: ambient
   - Mobile phase: methanol/acetonitrile/phosphate buffer (21/14/65 by vol)
   - Flow rate: 2 mL/min
   - Wavelengths: 195 and 254 nm
   - Attenuation: for 195 nm, 0.2 A full scale; for 254 nm, 0.01 A full scale.

2. Pipet 300 μL of standard, control, or patient’s serum (see Note 4) into appropriately labeled 16 × 100 mm glass tubes.
3. Add 300 μL of working internal standard to the tube and vortex-mix for 15 s.
4. Add 2 mL of a chloroform/methanol (4/1 by vol) mixture and vortex-mix for 15 s.

Note: Evaluators F.M.S. and R.D. found that vortex-mixing the patients’ samples frequently resulted in the formation of an inseparable emulsion or large emulsion interface. They recommend, instead, solvent extraction with 4 mL of the chloroform/methanol mixture in 16 × 125 mm disposable tubes, then transferring 2 mL of the organic phase to the 12 × 75 mm glass tubes for evaporation. In their hands this modification minimized the emulsion problems.

5. Cover tubes with Parafilm and centrifuge at 2500 rpm for 10 min.
6. Remove the aqueous (upper) layer and discard.
7. Transfer approximately 1 mL of the chloroform (lower) layer to a clean 12 × 75 mm glass tube.
8. Evaporate in a 55 °C water bath for 5 min under a fume hood.
9. Reconstitute the dry residue with 100 μL of mobile phase and vortex-mix for 60 s, until completely dissolved.

Note: Evaluators F.M.S. and R.D. found that these residues of serum-based controls and patients' samples, when reconstituted with 100 μL of mobile phase, consistently produced a turbid solution. They recommend the use of a column inlet filter (e.g., Rheodyne Model 7302 Column Inlet Filter) or precolumn (e.g., Fälliguard LC-18, Supelco cat. no. 5-9232) to extend the life of the analytical column.

10. Inject 20 μL of the reconstituted sample.

11. To shut down the chromatographic system, pump filtered de-ionized water for 15 min, then filtered methanol for 15 min, leaving methanol on the column.

Calculations (see Notes 1, 2 below)

1. Calculate the concentration of each drug in the unknown (unk) at 195 nm and at 254 nm as follows:

\[
\text{Conc of unknown, mg/L} = \frac{\text{PH drug (unk)/PH IS (unk)}}{\text{PH drug (WS)/PH IS (WS)}} \times C
\]

where \( \text{PH} \) = peak height, \( \text{IS} \) = internal standard, \( \text{WS} \) = working standard, and \( C \) = concentration of the corresponding drug in the working standard.

2. Confirm that the results of the quantifications at each wavelength agree (see Note 3 below).

3. Report the results determined at 195 nm, except for carbamazepine (see Note 3).

Quality Control

Use TheraChem Anticonvulsant/Theophylline Control (Fisher Scientific, cat. no. 2588-69) or equivalent. Establish mean and standard deviation values according to the usual laboratory protocol.

**Expected therapeutic ranges, mg/L:**
- Primidone, 5–15
- Phenobarbital, 20–40
- Phenytin, 10–20
- Carbamazepine, 4–8

**Notes:**
1. If a peak height is off-scale, the serum should be diluted with de-ionized water and the diluted sample carried through the extraction procedure. The result is multiplied by that dilution factor.
2. Linearity of response to concentration has been established within the following ranges (mg/L): primidone, 2–25; phenobarbital, 2–50; phenytin, 1–40; carbamazepine, 1–40.
3. Because the response of carbamazepine is greater at 254 nm than at 195 nm, it is preferable to report the 254-nm results for this drug. The response to primidone is very poor at 254 nm, so that the results at the two wavelengths will frequently not agree; report only the 195-nm results for primidone.
4. Plasma may be substituted if appropriate in-house studies verify the interchangeability of the specimens.

**Results and Discussion**

The role of therapeutic monitoring in patient care is well established. As an evolving clinical laboratory service, however, many areas remain unclear. The resolution of problems with accuracy in therapeutic drug monitoring awaits the establishment of definitive and (or) reference methods. Interlaboratory precision for these assays is similar to that of other clinical laboratory activities (3–7). The level of intralaboratory precision required and that being achieved have been documented (8).

"High-pressure" liquid chromatography methods for drug monitoring have been reviewed previously (2). The method presented here is intended to be easily transferable and flexible for meeting individual laboratory needs. The mobile phase may be adjusted to delay or accelerate the elution of individual drugs if appropriate. Some workers may degas the mobile phase components before blending; however, we noted no degradation of the mobile phase prepared and handled as described above.

For the purpose of this presentation, we analyzed each specimen within 24 h. Our experience, however, is that these samples are stable for as long as three days at 4 to 6 °C, or seven days or more at −20 °C. If analysis is to be delayed beyond 24 h, we recommend that the samples be frozen and that each laboratory document sample stability under their own conditions.

We decided not to use guard columns in this procedure. If such apparatus is introduced, the user must be alert to note changes, if any, in the performance of the method.

Run-to-run (different days) precision (CV) for the method described is: primidone 7% (mean 12.4 mg/L, n = 31); phenobarbital 5% (32.3 mg/L, n = 27); phenytin 7% (17.7 mg/L, n = 31); carbamazepine 7% (8.1 mg/L, n = 30). This precision is within the performance limits required for medical management (8).

Analytical recovery (Table 1) was established by dissolving pure drug in a small amount of methanol and diluting to a final volume with drug-free serum. Each sample was analyzed three times and the results averaged.

The following drugs in the expected therapeutic ranges do not interfere: gentamicin, tobramycin, procainamide (and its metabolite N-acetylprocainamide), lidocaine, theophylline, valproic acid, and ethosuximide. Ethosuximide is substantially removed during the extraction step; any remaining in the sample would elute just before primidone.

For some antiepileptic agents, a metabolite(s) may have antiseizure activity, in which case the metabolite should be quantified in addition to or instead of the parent drug. Phenytoin and phenobarbital are measured as the parent drug. Primidone should be monitored by quantifying the parent drug and its major metabolite phenobarbital. Although the carbamazepine 10,11-epoxide is considered by some to be the proper analyte for monitoring carbamazepine therapy, this is not widely accepted, and we did not study it.

The goal of antiepileptic therapy is to create a consistent concentration of drug and (or) its active metabolite(s) sufficient to prevent seizures but low enough to prevent undesired effects. Monitoring the concentrations of specific antiepileptic agents and (or) metabolites in blood assumes a stable relationship between concentrations in the circulating blood (total drug) and concentrations at active sites (free drug at receptor) (2).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug concn, mg/L</th>
<th>Added</th>
<th>Detected</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbazepine</td>
<td>5.0</td>
<td>5.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>10.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>20.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Phenytin</td>
<td>7.0</td>
<td>7.1</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>10.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.0</td>
<td>21.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>15.0</td>
<td>15.2</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>30.2</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td>39.0</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Primidone</td>
<td>5.0</td>
<td>5.3</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>7.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>14.8</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

*(Added/detected) × 100.*
Not only is it common practice to use antiepileptic agents in combination, but also one drug may affect the metabolism of the other(s). It is therefore desirable to quantify several agents in each sample, even if only one has been requested. For instance, phenobarbital should be checked in each sample submitted for phenytoin determination, because these two are frequently used together and phenobarbital has a marked influence on phenytoin metabolism. Likewise, phenobarbital, the major active metabolite of primidone, must be quantified in each sample submitted for primidone determination.

The methods used for quantifying antiepileptic agents include spectrophotometry, gas–liquid chromatography, enzyme immunoassays, radioimmunoassay, and "high-pressure" liquid chromatography. The underlying difference among these techniques is the way in which the drug of interest is separated for quantification. Spectrophotometric assays lack the sensitivity and specificity attainable by more recently introduced methods such as immunoassays and chromatographic techniques. Gas–liquid chromatography is used widely and has the advantage of simultaneous quantification of several agents with one injection; however, this technique at times requires that some derivative of an analyte be made and is limited by thermal instability of some drugs (e.g., carbamazepine). Enzyme immunoassay and radioimmunoassay are good techniques for quantifying drugs for which specific reagents are available. A separate analysis for each agent and (or) its metabolite(s) is usually required when using immunoassays. "High-pressure" liquid chromatography allows for simultaneous measurement of a mixture of analytes in one sample injection without the need for derivative formation or markedly high temperatures.

References

Editor's note: The reader is reminded that Selected Methods do not bear the official imprimatur of the Association. As detailed elsewhere (Clin. Chem. 19:1207, 1973), these methods are offered here for criticism by the world community of users, and will be revised appropriately before being collected into a bound volume. Selected Methods of Clinical Chemistry. The latest volume in this series was published by the Association in 1983. The Committee on Selected Methods attempts to select and evaluate methods that seem durable and generally useful, which have been checked by several Evaluators to determine both advantages and disadvantages. Thus sufficient information is provided to enable the user to know what to expect. Designation as a Selected Method does not imply superiority in all respects, nor is the procedure a selected proposed reference method unless it is so designated or has been developed as such by the AACC Standards Committee. The published procedure should be superior in terms of evaluation and thus of accurately describing to the user the characteristics of the method.