Felbamate Measured in Serum by Two Methods: HPLC and Capillary Electrophoresis

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We have developed two methods for determining serum concentrations of felbamate, a new anticonvulsant drug. The first method is based on protein precipitation with acetonitrile, followed by HPLC. The between-run CV for this method is 5.7% (mean 55 mg/L), and the linearity extends from 5 to 175 mg/L. Results by this method compared well with those by an HPLC method based on chloroform extraction (r = 0.98, n = 21). In the second method, based on micellar electrokinetic capillary chromatography, the drug is measured by capillary electrophoresis with direct injection of serum. This method can be completed in 5 min compared with 12 min for the HPLC method, and there is no need for sample extraction. The between-run CV is 5.2% (mean 58 mg/L) and the linearity range is 5–160 mg/L. Results of this direct method correlated well (r = 0.98, n = 37) with those by the HPLC assay. The mean trough serum concentration of felbamate in 123 patients taking this drug was 44.9 mg/L (range 12–129 mg/L).

Indexing Terms: anticonvulsant drugs/drug assay/monitoring therapy/methods comparison

Felbamate (Felbatol; 2 phenyl-1,3-propanediol dicarbamate) is a new anticonvulsant drug approved recently for the treatment of partial seizures (1). It is also effective against Lennox–Gastaut syndrome (2). Felbamate appears to have a wide therapeutic index. Half of the amount taken is excreted in urine unchanged; two hydroxy derivatives and other unidentified metabolites are also found in urine. Although the therapeutic range for this drug has not been well established, the serum concentrations for patients on monotherapy are 15–135 mg/L (2–4).

Because immunoassay methods are not yet available for analysis of felbamate, we report here two assay methods we developed. The first is based on HPLC, the second on capillary electrophoresis (CE). Unlike previous HPLC methods for felbamate (5–7), our HPLC method does not require solvent extraction, and is thus more suitable for routine analysis. However, like other HPLC assays, our method requires organic solvent in the mobile phase, a somewhat lengthy separation time, and skill.

Our CE method exemplifies analyses for drugs and endogenous substances by this new technique (8). Felbamate is a nonionized molecule; however, as Terrabe et al. (9) have shown, such compounds can be separated and measured by CE on the basis of micellar electrokinetic capillary chromatography (MECC). In this technique (10, 11), a surfactant is added to the buffer at a concentration exceeding the critical micellar concentration. Neutral and hydrophobic compounds are separated on the basis of their partition between the aqueous phase of the buffer and the micellar pseudophase. The separation is analogous to that of the reversed phase in HPLC.

Evenson and Wiktowicz (12) have demonstrated the potential of MECC to analyze many drugs, provided a clean-up step is performed first. Because the surfactant used in MECC has the added advantage of solubilizing the serum proteins (13), we devised a simple way to determine serum felbamate by this method without sample treatment other than adding an internal standard. This method does not require sample extraction or protein removal, is rapid (<5 min) and fully automated, does not require organic solvents, and is less expensive than immunoassays or HPLC.

Materials and Methods

HPLC

Equipment. A Model 110 A pump (Beckman Instruments, Fullerton, CA), was used to deliver the pump solvent (acetonitrile, 125 mL/L of phosphate buffer (20 mmol/L), pH 6.1) at 1.8 mL/min through a 125 × 4 mm (i.d.), C8 (5-μm particles) cartridge column (E. Merck, Gibbstown, NJ). The effluent was detected at 205 nm with a Model Spectro Monitor II detector (Laboratory Data Control, Riviera Beach, FL) at 0.020 A. The sample was introduced through a 10-μL loop. New columns are initially washed with 100 mL of acetonitrile before use.

Preparation of calibrators. Stock solution (250 mg/L) of felbamate (Carter-Wallace, Cranbury, NJ) was prepared by dissolving 250 mg in 100 mL of methanol and bringing the volume to 1 L with water. The stock solution was further diluted to make appropriate concentrations and treated identically to patients’ samples. Because the stock calibrator solution has a tendency to crystallize upon refrigeration, it was warmed to 37°C and sonicated before making new working calibrators.

Controls. We added stock felbamate to a commercial serum pool to a final concentration of 50 mg/L.

Procedure (acetonitrile deproteinization). We added 100 μL of serum, calibrator (50 mg/L), or control to 200 μL of acetonitrile containing 12 mg/L acetoacetanilide (Eastman Kodak, Rochester, NY) as an internal stan-
standard, centrifuged the mixture at 14,000g for 15 s, and injected a 50-μL aliquot of the supernate into the column. Calculations were based on peak heights.

Chloroform extraction. For comparison studies only, we extracted 21 samples by adding 50 µL of serum or calibrator to 1.0 mL of chloroform. After vortex-mixing for 30 s, the top layer was removed and discarded. The chloroform layer was evaporated in small vials. The contents of the vials were reconstituted with 100 µL of 500 mL/L acetonitrile containing 7 mg/L acetocetanilide.

Capillary Electrophoresis

Equipment. A Model 2000 CE instrument (Beckman Instruments) was set at 214 nm and 35°C. The capillary was 500 mm × 50 μm (i.d.). The running buffer was boric acid (100 mmol/L, adjusted to pH 8.4 ± 0.1) with 2 mol/L NaOH and contained 55.4 mmol/L sodium dodecyl sulfate (SDS). The SDS was dissolved in the buffer by sonication for 5 min. Between runs, the capillary was washed for 1 min with 100 mmol/L phosphoric acid, and filled for 1 min with the running buffer. Daily, the buffer containers were emptied, cleaned, and filled with the running buffer, and the capillary was washed for 3 min with each of the following: NaOH (2 mol/L), phosphoric acid (100 mmol/L), and the running buffer. The instrument was set at a fixed current of 38 mA. The voltage reading was ~16.5 kV. The sample was introduced by pressure-injection for 5 s.

In some of the preliminary work, we also used a homemade instrument constructed from a Model EL power supply (Glassman, Whitehouse Station, NJ) and a Model 4225 Unicam variable-wavelength detector (ATI Instruments, Boston, MA). For safety, the electrical connections to the electrodes were terminated at the cell cover that housed the buffer containers. Thus, when the cover was lifted, there was no potential at the containers or at the electrodes. We used this instrument mainly to study the effect of different wavelengths on the peak height.

Internal standard. Acetocetanilide (200 mg) was dissolved in 25 mL of methanol and diluted to 1 L with water.

Calibrators. The stock calibrator solution of the HPLC method was diluted in a serum pool free of felbamate or in a commercial serum control to make a 50 mg/L single-point working calibrator.

Linearity and recovery studies. A special stock calibrator of felbamate, 600 mg/L in 100 mL/L methanol, was prepared. This calibrator was further diluted in water, saline (NaCl 7 g/L), or a serum pool from healthy individuals.

Procedure. Equal volumes (typically 25 µL) of serum, calibrator, or control and the internal standard were mixed and introduced into the instrument.

Calculation. The calculation was based on peak height, by comparison with a single-point calibrator.

Interference studies. A serum sample was supplemented with the drugs listed in Table 1 and assayed in the presence and absence of felbamate.

### Table 1. Drugs that do not interfere with felbamate analysis.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. tested, mg/L</th>
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<tbody>
<tr>
<td>Acetaminophen</td>
<td>120</td>
</tr>
<tr>
<td>N-Acetylsalicylamide</td>
<td>15</td>
</tr>
<tr>
<td>Amikacin</td>
<td>37</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>18</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0.004</td>
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<tr>
<td>Disopyramide</td>
<td>9</td>
</tr>
<tr>
<td>Ethosuximide</td>
<td>158</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>10</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>51</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>39</td>
</tr>
<tr>
<td>Primidone</td>
<td>15</td>
</tr>
<tr>
<td>Procaainamide</td>
<td>12</td>
</tr>
<tr>
<td>Quinidine</td>
<td>7</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>68</td>
</tr>
<tr>
<td>Theophylline</td>
<td>51</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>195</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>45</td>
</tr>
</tbody>
</table>

Results and Discussion

HPLC

Felbamate is a phenolic compound with strong ultraviolet absorption at ~205 nm. This strong absorptivity of the drug and its high concentrations in serum (15–135 mg/L) make it suitable for determination by acetocetanilide deproteinization or direct injection onto the HPLC column (14). However, because of the presence in serum of numerous endogenous compounds as well as the possibility that many other drugs that absorb at this wavelength will be present, the separation of felbamate from such substances is critical. For a study of felbamate in dogs, Clark et al. (15) described an assay based on acetocetanilide deproteinization and the use of an HPLC C18 column. However, they made no attempt to separate the drug from potentially interfering compounds that might be present in human serum. Here, using a different type of column, a C8 cartridge, and the specified conditions, we were able to separate felbamate and the internal standard from the interfering compounds.

Acetocetanilide is expensive and environmentally hazardous; therefore, we kept its concentration in the mobile phase to a minimum, using an octyl cartridge column. We tested the mobile phase at a pH range of 2.2–7.6. We chose pH 6.1 because at this pH none of the common drugs listed in Table 1 interfered with felbamate. Also, in examining 10 different pools of serum from hospitalized patients we encountered no endogenous or exogenous interfering substances.

The retention times for the internal standard and felbamate were ~8.4 and 12.5 min, respectively (Fig. 1). Most of the sera did not contain any peaks eluting after felbamate. Occasionally, such compounds appeared on the chromatograms as wide peaks; they did not affect patients’ results but did increase the analysis time. To prevent interferences from affecting the felbamate peak, we kept the acetocetanilide concentration in the mobile phase relatively low, as specified, allowing a baseline
separation between the felbamate and the internal standard as in Fig. 1. Increasing this concentration to speed up the analysis time led to coelution of interfering substances with the felbamate peak.

The assay response was linear with felbamate concentration from 5 to 175 mg/L (Fig. 2). The mean recovery of 50 mg/L felbamate added to serum pools was 98.4% (n = 5, range 94.7–100%), indicating that a small amount of felbamate coprecipitated with the proteins. For many of the methods involving deproteinization by acetonitrile, the volume ratio of serum to acetonitrile is 1:1. However, this ratio is not adequate to remove serum proteins completely (16); it also lowers the recovery of felbamate and shortens the column life. The minimum detectable concentration (3 SD of the baseline noise) was 3 mg/L. The CV for 10 injections (within-run at 25 mg/L) was 3.2%, for 22 samples (between-run) over 30 days, 5.7% (mean 55 mg/L), and for 15 duplicate analyses over 14 days, 4.6%. We compared this method (y) with the chloroform extraction method (x) for 21 samples (Fig. 3). The regression analysis yielded y = 0.99x + 1.1 mg/L (r = 0.98, S_yx = 0.039). The chloroform extraction yielded samples with fewer peaks in the early but not in the later part of the chromatogram.

CE

Because this drug is a neutral compound, we chose MECC for the separation. In this technique, the surfactant SDS forms micelles with negative charges on the outside surface (10, 11). The neutral molecules partition and migrate with the micelles (10, 11) toward the cathode.

Direct injection of serum (a few nanoliters) into the capillary is an attractive approach for analysis of drugs because of its simplicity and suitability for automation; however, in practice, this poses several problems. The absorptivity of the wavelength range (200–214 nm) is not specific for any class of compounds. Many substances absorb light at this region and can interfere with felbamate or the internal standard. Serum proteins, which are present in high concentrations, can mask the absorbance of felbamate or bind the drug, leading to a low recovery or altered migration. Thus the separation becomes a challenge to prevent these compounds from interfering with the felbamate and the internal standard peaks. The selection of the optimum conditions for the assay such as pH, ionic strength, SDS concentration, voltage, and capillary length are critical for good separation. As shown in Fig. 4, felbamate was separated from other compounds in the serum, and the assay was rapid. The migration times for the internal standard and felbamate were 3.9 and 4.3 min, respectively.

Initially, we studied the optimum conditions for separating felbamate from other compounds present in serum. We evaluated different buffers at different pH and molarities. We also tested the concentration of SDS from 5 to 40 g/L. Increasing the SDS concentration to >30 g/L increased the peak height but caused the felbamate peak to elute between serum proteins, appearing as a sharp peak between broad peaks. Increasing the ionic strength of the buffer improved the separation but increased the migration time. Increasing the buffer pH also increased the separation time. An optimum current (33–40 μA) was important for obtaining a short analysis time.
Because of its effect on current conductivity and stacking properties, the sample matrix, especially its ionic strength, affects the separation in capillary zone electrophoresis (CZE) (16, 18). Furthermore, ∼25% of the felbamate is bound to serum proteins. If this binding is not dissociated during the assay, recovery of the drug may be low. The mean recovery of 50 mg/L felbamate added to a serum pool (n = 5) was ∼106% of the standard prepared in isotonic saline, and 125% of the standard prepared in water. These data indicate that felbamate dissociates from serum proteins. More important, they demonstrate that the sample matrix affects peak height in MECC as in CZE (16, 18). Thus, we prefer to prepare the working standard in serum. The minimum detectable concentration (3 SD of baseline signal) was ∼4 mg/L.

The assay was linear between 5 and 160 mg/L (Fig. 5). The CVs are given in Table 2. A thorough wash with phosphoric acid (100 mmol/L) between runs to remove any proteins adsorbed to the capillary walls is critical for good reproducibility (16). We compared results for 37 samples assayed by this method with results from HPLC. The correlation between the two methods was good (r = 0.98, S_yx = 0.026) (Fig. 6).

Routine therapeutic drug monitoring is performed in the clinical laboratory mainly by immunoassays. However, there being no commercial immunoassays for felbamate, CE/MECC and HPLC present good alternative techniques. In both of these methods the separation is based on partition between two phases, and their precision and linearity are comparable. However, the cost per test for CE is much less than that for the HPLC or immunoassays: The capillary in CE is much less expensive than the HPLC column and can be used for >100

**Table 2. Felbamate assay precision.**

<table>
<thead>
<tr>
<th>Mean, mg/L</th>
<th>n</th>
<th>CV, %</th>
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</thead>
<tbody>
<tr>
<td>Within-run</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>Between-run</td>
<td>58</td>
<td>21</td>
</tr>
<tr>
<td>Duplicate</td>
<td>—</td>
<td>20</td>
</tr>
</tbody>
</table>

Fig. 4. CE electropherograms of: (A) calibrator in saline, containing felbamate (F) 50 mg/L and phenobarbital (P) 45 mg/L, and (B) serum from a patient taking felbamate (53 mg/L) and phenobarbital (18 mg/L).

The plate number for the felbamate peak, based on the formula described by Vinther and Soeberg (17) for the capillary, was ∼150 000, offering the needed resolution for separation of the numerous endogenous and exogenous compounds in serum. Under the described conditions the serum proteins migrated slowly enough not to appear on the electropherogram within the 5-min assay time. The common drugs listed in Table 1 did not interfere in the assay, and we encountered no endogenous interferences in five different serum pools examined. One of the drugs that eluted close to felbamate was phenobarbital (Fig. 4)—a drug that can be measured by this same method if serum-based calibrators are used.

Fig. 5. Linearity of felbamate measurement in serum by CE.

Fig. 6. Correlation of felbamate measurement (mg/L) by HPLC and CE: CE = 1.14 HPLC + 2.67 (r = 0.98, n = 37, S_yx = 0.026).

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samples. Also, the separation time in CE is about half that in HPLC, and no protein removal or solvent extraction is needed. The simplicity of the CE method illustrates the potential of CE for therapeutic drug monitoring as well as for analyzing endogenous substances. The final choice between CE or HPLC for felbamate analysis depends largely on the experience of the operator and the availability of the instruments in the laboratory.

The mean trough value concentration (± SD) of felbamate in 123 samples from patients on polytherapy was 44.9 ± 22.5 mg/L (range 12-129 mg/L). These concentrations are consistent with previously reported values for such patients (2-4). Given that most of our patients are on polytherapy, it is difficult to study or comment on the therapeutic window at this time.

We thank Beckman Instruments for providing the CE instrument.

References