Drug Monitoring and Toxicology: A Simple Procedure for the Monitoring of Felbamate by HPLC–UV Detection

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Abstract

This article describes a simple isocratic high-performance liquid chromatographic (HPLC) method with UV detection for the determination of felbamate in the serum of patients with epilepsy. Sample preparation requires only protein precipitation with a single-step methanol extraction. After centrifugation, the resulting supernatant was injected directly onto the HPLC system. Separation was achieved by reversed-phase HPLC, using a 5-µm Microsorb-MV C18 column (250 x 4.6 mm) connected to a Silica C18 guard column (20 x 4.6 mm) and a mobile phase consisting of a mixture of phosphate buffer (pH = 6.9, 0.05 M), methanol, and acetonitrile (64:18:18, v/v/v). The flow rate was at 1.0 mL/min and column temperature was set at 35°C. Quantitation was performed by measurement of the UV absorbance at a wavelength of 210 nm. Calibration curves were linear over a range of 2–200 mg/L, which covered the proposed range of 50–150 mg/L for reference. Both within-run and between-run precision were lower than 5%. Recoveries ranged between 97% and 105% for spiked and pooled samples. No interferences with other common antiepileptic drugs (except zonisamide) were observed. The method requires only 100 µL of serum or less. It is simple and fast (sample preparation and analysis time) and suitable for routine clinical use.

Introduction

Felbamate is described chemically as 2-phenyl-1,3-propanediol dicarbamate. It is used to treat partial seizures (with and without generalization) in adults and partial and generalized seizures associated with Lennox-Gastaut syndrome in children (1,2). However, increased risks of potentially fatal aplastic anemia (3) and liver failure (4) limit the drug’s usage to severe refractory epilepsy. The mechanism by which felbamate exerts its anticonvulsant activity is unknown, but in animal test systems designed to detect anticonvulsant activity, felbamate has properties in common with other marketed anticonvulsants. After oral administration, the bioavailability of felbamate has been estimated to be at least 90% (5), and peak serum concentrations have been attained within 4 h (6). Serum felbamate concentrations of 30–60 mg/L have been associated with therapeutic doses (7–9). However, a retrospective study suggested target trough concentrations of 50–110 mg/L for optimal seizure control (10). Recently, a reference range of 50–150 mg/L was proposed by the Toxicology Laboratory of the University of Virginia (Charlottesville, VA). Although toxicity studies have been reported in mice and rats (11), the toxic and lethal levels are yet to be established.

Therapeutic drug monitoring of antiepileptic drug (AED) serum concentrations is helpful to physicians in evaluating patient compliance with treatment, in providing guidance to achieve well-tolerated and effective AED dosing, and in identifying drug-drug interactions when drugs are given as polytherapy. Retrospective studies noted that significant interactions were observed when felbamate was administered with other antiepileptic drugs (12–18), including valproate, phenytoin, carbamazepine, phenobarbital, lamotrigine, gabapentin, and levetiracetam. Because of its complex but poorly understood mechanism(s) of interaction with other drugs, dosage adjustment is essential and requires constant monitoring of felbamate levels in serum.

Serum felbamate is generally measured by high-performance liquid chromatographic (HPLC) assays (19–24). In some HPLC assays, multi-step extraction techniques and extensive sample pretreatment are used (19–22). Simple HPLC procedures based on direct HPLC injection after sample deproteinization or even without sample pretreatment have been reported (23,24). However, short-comings of these methods include the need for a large sample size (500 µL) (24), and they are, therefore, not suitable for use in pediatric patients or in a gradient elution (23) to eliminate interferences. Previously, HPLC analysis of serum felbamate ordered by the physicians at the Cincinnati Children’s Hospital Medical Center (CCHMC) was performed at the reference laboratory. The results turnaround time was not always satisfied, and service of therapeutic drug monitoring was, therefore, lagging. The need for a quick measurement of felbamate in serum samples in a sim-
plified manner and the need for a cost-effective procedure prompted the development of a rapid HPLC assay, which was adapted and modified from the existing methods, used here for measuring other drugs such as carbamazepine, lamotrigine, and oxcarbazepine. Here, a simple HPLC method is described for determination of felbamate concentrations in a small volume of serum that is suitable in pediatric practice. Sample preparation involves precipitation of proteins only with a single step methanol extraction, rather than the more complex liquid–liquid extraction procedure. LC is performed using an isocratic elution. Trimethadione internal standard and felbamate elute in less than 7 min, and the whole procedure requires 9 min for completion.

Experimental

This study was approved by the Institutional Review Board of the CCHMC. In this study, 30 de-identified samples ordered for serum concentrations of felbamate were included for comparison between the current method and reference method. To study potential interference, 24 de-identified samples submitted for the testing of other drugs were also analyzed. These drugs included: acetaminophen; salicylate; ibuprofen; valproic acid; carbamazepine, carbamazepine-epoxide, and hydroxy metabolites; phenytoin and metabolite; phenobarbital; topiramate; oxcarbazepine and its monohydroxy metabolite; lamotrigine; levetiracetam; methsuximide and normethsuximide; zonisamide; and gabapentin.

Apparatus

The HPLC system (SpectraSYSTEM, Thermo Separation Products, Fremont, CA) consisted of a model P4000 binary pump, a model AS3000 automatic sample injection device equipped with a 100-µL sample loop, and a model UV2000 dual-wavelength detector. The output signal was monitored and processed using ChromQuest software (Thermo Separation Products). The analytical column was a 250-mm x 4.6-mm Microsorb-MV C18 column (Varian, Lake Forest, CA) with 5-µm spherical particles. A Silica C18 20-mm x 4.6-mm guard column (Hamilton, Reno, NV) was used to protect the analytical column. The mobile phase consisted of acetonitrile/methanol/0.05 M phosphate buffer at 18:18:64 (v/v/v), pH adjusted to 6.9 using orthophosphoric acid. Phosphate buffer was prepared by dissolving 4.42 gm potassium phosphate monobasic and 3.99 gm potassium phosphate dibasic trihydrate in 1 L of water. The mobile phase was filtered before use through a 0.2-µm (47-mm diameter) MAGNA nylon filter (GE Water & Process Technologies, Minnetonka, MN) under reduced pressure. The dual-wavelength detector is needed to perform the analysis, because the first wavelength of 210 nm is for monitoring the felbamate and trimethadione, and the second wavelength of 286 nm is for detecting interference such as zonisamide.

Trimethadione and felbamate were obtained from Sigma (St. Louis, MO). Other chemicals used were analytical grade or HPLC grade. The stock solution of felbamate (200 mg/L) and working solution of internal standard (400 mg/L) were prepared in methanol. Serum free of felbamate was pooled, stored at −20°C, and centrifuged to remove clots. A series of six calibrator solutions, ranging from 2 to 200 mg/L, were prepared using the felbamate stock solution with the appropriate volume of methanol to final concentrations of 2, 10, 25, 50, 100, and 200 mg/L. Four control solutions were prepared in this manner for 20, 40, 80, and 160 mg/L. Another stock solution of felbamate (1 mg/mL) was prepared by dissolving 25 mg felbamate in water. The felbamate/water solution was used to prepare another set of quality control (QC) samples (20, 40, 80, and 160 mg/L) by mixing appropriate amounts of felbamate/water solution with pooled serum.

Methods

Serum calibrators or controls were prepared by transferring 100 µL of pooled serum into respective microcentrifuge tubes containing 100 µL of calibrator or control solution, and capped and stored at −20°C until analysis. After samples were thawed, 100 µL of internal standard solution was added to each tube followed by 200 µL of methanol. Patient or pooled QC samples were prepared by transferring 100 µL of patient or pooled QC serum into respective microcentrifuge tubes; 100 µL of internal standard solution was then added to each tube, followed by 300 µL of methanol. The tubes were capped and vortex mixed for 2 min and then centrifuged for 5 min at 10,000 rpm. The supernatant was transferred to an autosampler vial, capped, and 20 µL was then injected onto the column. Single injection was analyzed from each sample extract. Ratios of the peak height of the calibrator divided by the peak height of the internal standard were used to establish a calibration curve and to quantify the felbamate from the calibration curve.

The linearity of the method was evaluated by analyzing

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time (min)</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>&lt; 4.0</td>
<td>-</td>
</tr>
<tr>
<td>Levetiracetam</td>
<td>&lt; 4.0</td>
<td>-</td>
</tr>
<tr>
<td>Salicylate</td>
<td>&lt; 4.0</td>
<td>-</td>
</tr>
<tr>
<td>2-Ethyl-2-phenylmalonamide</td>
<td>4.32</td>
<td>0.79</td>
</tr>
<tr>
<td>Trimethadione</td>
<td>5.47</td>
<td>1.00</td>
</tr>
<tr>
<td>Felbamate</td>
<td>6.46</td>
<td>1.18</td>
</tr>
<tr>
<td>Zonisamide</td>
<td>6.51</td>
<td>1.19</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>7.51</td>
<td>1.30</td>
</tr>
<tr>
<td>Monohydroxycarbamazepine</td>
<td>8.84</td>
<td>1.62</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>9.56</td>
<td>1.75</td>
</tr>
<tr>
<td>Normethsuximide</td>
<td>10.98</td>
<td>2.01</td>
</tr>
<tr>
<td>Carbamazepine-epoxide</td>
<td>11.64</td>
<td>2.13</td>
</tr>
<tr>
<td>Methsuximide</td>
<td>18.27</td>
<td>3.34</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>22.41</td>
<td>4.10</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>25.08</td>
<td>4.59</td>
</tr>
<tr>
<td>Valproate</td>
<td>n.d.*</td>
<td>-</td>
</tr>
<tr>
<td>Topiramate</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>n.d.</td>
<td>-</td>
</tr>
</tbody>
</table>

* n.d.: not detected.
pooled sera to which six calibrator solutions were added, and by least-squares linear regression analysis. This experiment was performed three times over a period of two months. Each serum calibrator was analyzed in duplicate and concentrations were calculated from a six-point calibration curve.

The absolute analytical recovery was determined at a concentration for felbamate (50 mg/L) and for internal standard at the concentration which was used during the analysis of the serum samples (400 mg/L). Absolute analytical recovery was determined by comparing the average peak height for five extracted serum samples spiked with felbamate and internal standard with that for five aqueous solutions of felbamate and internal standard of identical concentrations in which serum was replaced by water.

Standard solutions of several commonly prescribed drugs and their metabolites were injected to check for possible interference (Table I). Blank plasma from 10 pooled sera was tested for endogenous interferences. Furthermore, serum samples from patients not taking felbamate and treated with commonly prescribed drugs were analyzed to check for potential interferences.

The within-run accuracy and precision were evaluated using six determinations per control. The deviation of the mean from the true value served as the measure of accuracy. Between-run accuracy and precision were evaluated by carrying out 12 independent determinations for each of 4 controls over a period of 2 weeks. Each control was analyzed in duplicate.

Further method validation was carried out by four different analysts using six patient samples. Patient samples were stored at 4°C over a period of two weeks; each sample was analyzed in duplicate. The stability of felbamate in serum sample was also assessed by storage of six patient samples at 4°C over a period of two weeks and at room temperature in the light for four days and seven days; values obtained were compared with fresh samples.

Accuracy of the method was further examined by measuring the concentrations of felbamate in 30 de-identified patient samples. The results obtained by this method were compared with results from the reference laboratory.

Results and Discussion

The felbamate assay was adapted from the routine method protocols, which measure levels of lamotrigine, oxcarbazepine, zonisamide, or carbamazepine. Although the flow rates and column temperatures are different among these method protocols, the same reversed-phase C18 column and same mobile phase are used. Using a single analytical column and a single mobile phase for multiple drug measurements can help managing turnaround time more efficiently and avoid column and mobile phase changes between different methods. Several commercially available compounds were investigated, and the trimethadione was found to be the best fit for an internal standard. As felbamate is a dicarbamate, quantitative extraction was obtained previously by using an acetonitrile protein precipitation step (18). It was found, however, that the same extraction efficiency was achieved with the use of methanol. The methanol extraction procedure in the felbamate assay is described here because of a desire to use similar extraction protocol for multiple anticonvulsant drugs.

Using the sample preparation protocol and chromatographic conditions described in Methods, a well-resolved and satisfactory separation of trimethadione internal standard (retention time: 5.5 min) and felbamate (retention time: 6.5 min) was obtained at a flow rate of 1 mL/min and column temperature at 35°C. A typical chromatogram monitored at a wavelength of 210 nm is presented in Figure 1. These two compounds resolved without any overlapping of their peaks or ambiguity in identification. Both compounds were eluted within 7 min. The effect of potential interference from other commonly used drugs was evaluated. The list of compounds and their retention times is given in Table I. No discernible peak (except zonisamide peak) from these compounds interfered with trimethadione and felbamate peaks. Lamotrigine, 5-(4-hydroxyphenyl)-5-phenylhydantoin, and monohydroxy carbamazepine were observed from 7 to 9 min. Therefore, the time required for completion of the assay was set for 9 min. An extra 2 min was found to be necessary to avoid the late eluting peaks of the extraneous compounds (Table I) that might be present in the serum samples, which would elute in the next injection.

Routinely, standards are prepared by spiking drug-free serum with the anticonvulsant drug and stored frozen in aliquots at -20°C. The drug is extracted and the serum proteins precipitated using methanol; the solvent fraction, after separating from the protein precipitate, is used for analysis. Recoveries of trimethadione and the felbamate were 101.4 ± 0.4% (mean ± standard deviation) and 101.2 ± 0.3%, respectively. Recoveries of both compounds spiked in serum were > 100%; this reflects the small volume displacement created by the precipitation of protein by methanol. However, the ratio of concentrations of felbamate and trimethadione remains constant regardless of the variations in matrix conditions. In this study, a calibration curve was prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of felbamate and trimethadione.
A maximal level of 171 mg/L has been observed in > 250 patient specimens for a routine felbamate test, which was reported by the Laboratory Administration at CCHMC. The validated linearity up to 200 mg/L for the method described here makes this method applicable across the wide range of serum concentrations found for patients receiving felbamate. The current method validation was based on the Guidance for Industry Bioanalytical Method Validation (25) published by the Food and Drug Administration. The following conditions were met in developing a calibration curve: accuracy within 80–120% of the target value for the lower limit of quantitation (LLOQ) and 15% deviation of standards other than LLOQ from nominal concentration. The current method was linear between the concentrations of LLOQ (2 mg/L) and the upper limit of quantitation (ULOQ, 200 mg/L). The regression equation was \( y = 0.01194x + 0.00286 \), where \( y \) is peak-height ratio of felbamate to that of trimethadione and \( x \) is the concentration of felbamate (in mg/L); the corresponding correlation coefficient was 0.999. The results show an excellent correlation between the peak-height ratios of felbamate/trimethadione and concentrations of felbamate. The coefficients of variation (CV) averaged 1.9% and 10.1% for felbamate at ULOQ and LLOQ, respectively. Analytical performance at LLOQ met criteria of precision of < 20% and accuracy within 80–120% of the target value. The LLOQ produced a peak 10 times as great as the baseline noise of a serum blank. The limit of detection (LOD) was estimated at a signal-to-noise ratio of 3:1 by diluting the LLOQ samples with pooled serum; the LOD was found to be < 0.6 mg/L.

The assay results for spiked and pooled samples are shown in Table II. There is no significant difference between results obtained using spiked and pooled serum (\( P < 0.0001 \)). It appears that there is no matrix issue in this assay. The accuracy was < 5% for all concentrations. The minimal deviation of the mean from the true value indicates the excellent accuracy of the method. The within-run precision was < 3% of CV, confirming good precision of the method. Between-run precision of felbamate demonstrated CV values of < 5%. Overall, the percentage recovery of felbamate ranged from 97 to 105%, indicating the consistent, precise, and reproducible extraction efficiency of the method. The current method can be performed by any trained person. Table III gives details of the method precision.
of these samples analyzed by four analysts who are medical technologists. All precisions were similar, with none of the CVs being > 4%.

Further method validation was performed; patient samples containing felbamate were diluted with pooled serum and assayed. These results are shown in Table IV. All the results were within 95–105% of anticipated concentrations, and similar results were observed upon serial dilutions of the high control from 160 to 20 mg/L. Thus, there did not appear to be matrix interferences compromising the accuracy of the method. The method is optimized for 100 µL of serum sample, but it can be done using a sample of ≥ 25 µL without compromising its accuracy. There was no evidence for loss of felbamate stored at 4°C for at least two weeks (P < 0.0001). Felbamate appeared stable at room temperature in the light for at least seven days (P < 0.0001), enabling postal samples to be accepted for analysis. Figure 2 illustrates a comparison between the current method and reference method. The correlation between the two methods was good; the linear regression statistics indicated an r value of 0.997 (P < 0.0001). The linear regression equation for correlation was y = 1.002x + 0.871 with a standard error of 2.63, where y is the current method and x is the reference laboratory method.

To study potential interference, 24 de-identified patient samples submitted for the testing of various therapeutic drugs were analyzed. An apparent interference was observed with a sample which contained zonisamide. The zonisamide peak eluted at about the same retention time as the felbamate peak under the current chromatographic conditions. Zonisamide is detected at both 210 nm and 286 nm, while felbamate is detected at 210 nm. Therefore, the interference from zonisamide was easily identified. Nevertheless, the combination of felbamate and zonisamide for treatment has yet been suggested. Other drugs assayed at therapeutic concentrations that did not interfere with this method include acetaminophen; salicylate; ibuprofen; valproate; carbamazepine, carbamazepine-epoxide, and hydroxy metabolites; phenytoin and metabolites; phenobarbital; topiramate; oxcarbazepine and its monohydroxy metabolite; lamotrigine; levetiracetam; methsuximide and normethsuximide; and gabapentin.

Conclusions

This paper describes a robust, accurate, and reproducible method for the measurement of felbamate in human serum. The equipment needed is the same as that used to measure other anticonvulsants via HPLC and UV detection. Sample preparation is rapid and efficient, and recovery excellent. The method can measure felbamate in 25–100 µL of sample, which is ideal for pediatric samples. The current method uses a single step methanol extraction and avoids the use of more complex liquid–liquid extraction or solid-phase extraction procedures, which substantially decreases set-up time. The method uses a commercially available internal standard and has potential cost savings in the use of a single extraction process, a single column, and a single mobile phase.

References


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