DETERMINATION OF THE ANTICONVULSANT FELBAMATE IN PLASMA SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY
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1) DRUG PROFILE - FELBAMATE

Felbamate is 3-(carbamoyloxy)-2-phenylpropyl carbamate is an antiepileptic used individually or as an adjunct to other anticonvulsants for the treatment of partial seizures resulting from epilepsy. In vitro receptor binding studies suggest that Felbamate may be an antagonist at the strychnine-insensitive glycine-recognition site of the N-methyl-D-aspartate (NMDA) receptor-ionophore complex. Antagonism of the NMDA receptor glycine binding site may block the effects of the excitatory amino acids and suppress seizure activity. However, Felbamate does interact as an antagonist at the strychnine-insensitive glycine recognition site of the NMDA receptor-ionophore complex. It is official in USP and BP. Its empirical formula is C\textsubscript{11}H\textsubscript{14}N\textsubscript{2}O\textsubscript{4}. The structure of Felbamate is shown in Fig-1.

![Fig-1. Structure of Felbamate](image)

**Chemical name:** 3-(carbamoyloxy)-2-phenylpropyl carbamate

**Chemical formula:** C\textsubscript{11}H\textsubscript{14}N\textsubscript{2}O\textsubscript{4}

**Molecular weight:** 238

**CAS Registry Number:** 25451-15-4
2) PAST WORK ON THE CHROMATOGRAPHIC METHOD FOR FELBAMATE

A literature survey revealed only a few reported analytical methods in HPLC with UV$^{3-10}$ detector, spectrometric determination and LC-MS/MS were also reported$^{11}$ for the determination of Felbamate from biological fluids either singly or with their degradation products.

A simple high-performance liquid chromatographic method for the determination of Felbamate in dog plasma was proposed by Clark L. A et al$^{3}$. Separation was achieved on a Waters Resolve C18, 5 microns, 300 mm × 3.9 mm I.D. column with a mixture of mobile phase 0.01 M phosphate buffer, pH 6.8-acetonitrile-methanol (800:150:50, v/v/v).

Jacalaa et al$^{4}$ described an improved RP-HPLC method for the simultaneous quantitation of Felbamate and its three metabolites in adult and neonatal rat brain and heart tissue homogenates. Separation was accomplished on a Waters Resolve C$_{18}$, 5 µm, 300 mm × 3.9 mm I.D. column with a mobile phase consisting of 0.01 M phosphate buffer, pH 6.8—acetonitrile—methanol (800:150:50, v/v/v). The detection was made at 210 nm.

Clark LA et al$^{5}$ have developed an isocratic liquid chromatographic one extraction step and a separation by using column 150 mm × 4.6 mm I.D. Spherisorb ODS2, 3-µm. The detection was UV-absorbance at 210 nm.

A fast, simple and sensitive high performance liquid chromatographic (HPLC) and Gas chromatography method has been described by Gur P et al$^{8}$ for the determination of Felbamate in serum. The efficiencies of Felbamate extraction by both C18 solid-phase extraction (SPE) followed by HPLC and liquid-liquid extraction followed by gas chromatography (GC) were evaluated. The method employs a high-performance liquid chromatography unit equipped with a C18 reverse-phase cartridge
(3-microliters particle diameter, 3.2 x 40 mm), an acetonitrile/water gradient, and
detection at 210 nm by Behnke C. E et al\textsuperscript{9}.

Beata P et al\textsuperscript{10} proposed a sensitive HPLC method for the assay of Felbamate in
serum, by using consisting of mobile phase acetonitrile: water (1:4 v/v). The
chromatographic separation was achieved with NOVA-PAK C18 HPLC column.

Hansen et al\textsuperscript{11} proposed a sensitive method for the assay of Felbamate in mouse
plasma, tissue and human plasma by using a rapid liquid chromatography tandem
mass spectrometry (LC-MS/MS). Samples were chromatographed on an XBridge
Phenyl, 2.5 \( \mu \)m and 4.6 mm X 50 mm column.
3) EXPERIMENTATION AND RESULTS

A) MATERIALS

i) Instrumentation

The author had attempted to develop a liquid chromatographic method for the determination of Felbamate using an isocratic Shimadzu HPLC equipment consisting of two LC10AT VP pumps, VP CTO-10AS VP column oven, a Hypersil BDS C<sub>18</sub> column (4.6 ID X 250 mm, 5µ) and an SPD-10A variable-wavelength. Programmable UV detector was used for chromatographic separation. The detection of the compounds was monitored at 216nm. Data acquisition was done by using Class VP Software. The details of the instruments employed in the study are as follows:

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Brand/Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC System</td>
<td>Shimadzu</td>
</tr>
<tr>
<td>Microbalance</td>
<td>Metler toledo</td>
</tr>
<tr>
<td>Deep Freezer</td>
<td>Sanyo (-86°C) VIP Series</td>
</tr>
<tr>
<td>Vibramax</td>
<td>Heidolph</td>
</tr>
<tr>
<td>Vacuum pump</td>
<td>Millipore</td>
</tr>
<tr>
<td>pH meter</td>
<td>Metler toledo</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>LG</td>
</tr>
<tr>
<td>Micropipettes, Multipette and Micro tips</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>Vortexer</td>
<td>Spinix</td>
</tr>
<tr>
<td>Solid phase extraction chamber</td>
<td>Water's</td>
</tr>
<tr>
<td>Poly propylene tubes</td>
<td>Torson's</td>
</tr>
<tr>
<td>HLB 30mg/1CC cartridges</td>
<td>Oasis; Water’s</td>
</tr>
<tr>
<td>Water Purification System</td>
<td>Millipore</td>
</tr>
<tr>
<td>Ultra sonicator</td>
<td>Power Sonic510, (Hwashin Technology)</td>
</tr>
<tr>
<td>Nitrogen Evaporator</td>
<td>Caliper</td>
</tr>
</tbody>
</table>

ii) Drug and Internal standard

The working standard samples of Felbamate and Zidovudine were gifted by M/s Hetero drugs Ltd., Hyderabad.
### iii) Chemicals and solvents

Acetonitrile (HPLC grade)
Methanol (HPLC grade)
Milli-Q water
Potassium dihydrogen phosphate (AR grade)
Ortho phosphoric acid (GR grade)
HLB OASIS 30mg/1CC cartridges
Human plasma
0.45µ Membrane filter

The linearity range of the drug was checked with the Q-Test at 95 percent confidence limits which was found to be well within the acceptable limits.

<table>
<thead>
<tr>
<th>Calibration Curve Standards table</th>
</tr>
</thead>
<tbody>
<tr>
<td>Points</td>
</tr>
<tr>
<td>STD 1</td>
</tr>
<tr>
<td>STD 2</td>
</tr>
<tr>
<td>STD 3</td>
</tr>
<tr>
<td>STD 4</td>
</tr>
<tr>
<td>STD 5</td>
</tr>
<tr>
<td>STD 6</td>
</tr>
<tr>
<td>STD 7</td>
</tr>
<tr>
<td>STD 8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>For Lowest Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Module D1</td>
</tr>
<tr>
<td>Ratio Q1</td>
</tr>
<tr>
<td>Q 95%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>For highest Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Module D2</td>
</tr>
<tr>
<td>Ratio Q2</td>
</tr>
<tr>
<td>Q 95%</td>
</tr>
</tbody>
</table>
iv) **Stock solutions of the drug and the internal standard**

a) **Stock solution of the drug**

Weighed 100.847 mg of Felbamate working standard compound was transferred into a 5 mL volumetric flask, dissolved in a mixture of methanol and water (60:40) and then the volume made up mark with a further quantity of same solvent mixture. The stock solution was sonicated for 5 min, to get a concentration of 20 mg/mL. This stock solution of the drug was stored in a refrigerator at below 10°C.

b) **Stock solution of the internal standard**

Weighed 100.647 mg of Zidovudine working standard compound was transferred into a 5 mL volumetric flask, dissolved in a mixture of methanol and water (60:40) and then the volume made up mark with a further quantity of same solvent mixture. The stock solution was sonicated for 5 min, to get a concentration of 20 mg/mL. This stock solution of the drug was stored in a refrigerator at below 10°C.

c) **Internal standard dilution**

0.015 mL of Zidovudine stock solution (20 mg/mL) was transferred into a 10 mL volumetric flask and the volume made up with a mixture of methanol and water (60:40). The solution was prepared fresh dilution daily.

v) **15 mM phosphate buffer (pH 3.6± 0.1)**

Weighed 2.04 grams of potassium dihydrogen phosphate and transferred into a 1000 mL reagent bottle and dissolved in 1000mL of Milli-Q water. The above solution was filtered through 0.45µ membarane, sonicated for 5 minutes and its pH was adjusted to (3.6 ± 0.1) with ortho phosphoric acid solution. The solution was stored at room temperature and used within 3 days from the date of preparation.

vi) **Mobile Phase**
The mobile phase was prepared by mixing with 90 parts of 15 mM potassium dihydrogen phosphate (pH 3.6 ± 0.1) buffer and 10 parts of acetonitrile in a reagent bottle, shaken well and sonicated for 5 min. The solution was stored at room temperature and used within 3 days from the date of preparation.

vii) Diluent

600 mL of methanol was transferred into a 1000 mL reagent bottle and 400 mL of Milli-Q water was added to this, mixed well and sonicated for 5 min. The solution was stored at room temperature and used within 7 days from the date of preparation.

viii) Washing solution

100 mL of methanol was transferred into a 1000 mL reagent bottle, 900 mL of Milli-Q water was added, mixed well and sonicated for 5 min. The solution was stored at room temperature and used within 7 days from the date of preparation. This solution was used in solid phase extraction for washing step.

ix) Rinsing solution

500 mL of acetonitrile was transferred into a 1000 mL reagent bottle, 500 mL of Milli-Q water was added, mixed well and sonicated for 5 min. The solution was stored at room temperature and used within 7 days from the date of preparation. This solution was used for rinsing the injection needle of the HPLC instrument.

x) Calibration Curve dilutions (CC Spiking solutions)

The calibration curve dilutions were prepared from Felbamate stock solution as per the table given below in the concentration range of 49.88 to 7538.19 µg/mL using a mixture of methanol and water (60:40) as the diluent. These dilutions (CC spiking solutions) were subsequently used for spiking the screened blank plasma.
<table>
<thead>
<tr>
<th>Stock/SS ID</th>
<th>Stock/SS Conc. (µg/mL)</th>
<th>Stock/SS Volume (mL)</th>
<th>Made up to Volume (mL)</th>
<th>Final Conc. (µg/mL)</th>
<th>SS ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCST</td>
<td>20048.38</td>
<td>1.8800</td>
<td>5</td>
<td>7538.19</td>
<td>SS8</td>
</tr>
<tr>
<td>SS8</td>
<td>7538.19</td>
<td>4.0000</td>
<td>5</td>
<td>6030.55</td>
<td>SS7</td>
</tr>
<tr>
<td>SS7</td>
<td>6030.55</td>
<td>3.3500</td>
<td>5</td>
<td>4040.47</td>
<td>SS6</td>
</tr>
<tr>
<td>SS6</td>
<td>4040.47</td>
<td>2.5000</td>
<td>5</td>
<td>2020.24</td>
<td>SS5</td>
</tr>
<tr>
<td>SS5</td>
<td>2020.24</td>
<td>1.9000</td>
<td>5</td>
<td>767.69</td>
<td>SS4</td>
</tr>
<tr>
<td>SS4</td>
<td>767.69</td>
<td>2.6200</td>
<td>5</td>
<td>402.27</td>
<td>SS3</td>
</tr>
<tr>
<td>SS3</td>
<td>402.27</td>
<td>1.2400</td>
<td>5</td>
<td>99.76</td>
<td>SS2</td>
</tr>
<tr>
<td>SS2</td>
<td>99.76</td>
<td>2.5000</td>
<td>5</td>
<td>49.88</td>
<td>SS1</td>
</tr>
</tbody>
</table>

**Quality control dilution (QC Spiking solutions)**

The quality control dilutions (QC spiking solutions) from Felbamate stock solution were prepared as per the table given below in the concentration range from 50.68 to 5814.38 µg/mL using a mixture of methanol and water (60:40) as the diluent. These dilutions (QC spiking solutions) were subsequently used for spiking the screened blank plasma.

<table>
<thead>
<tr>
<th>Stock/SS ID</th>
<th>Stock/SS Conc. (µg/mL)</th>
<th>Stock/SS Volume (mL)</th>
<th>Made up to Volume (mL)</th>
<th>Final Conc. (µg/mL)</th>
<th>SS ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQST</td>
<td>20049.58</td>
<td>1.4500</td>
<td>5</td>
<td>5814.38</td>
<td>HQCSS</td>
</tr>
<tr>
<td>HQCSS</td>
<td>5814.38</td>
<td>2.7000</td>
<td>5</td>
<td>3139.76</td>
<td>MQCSS</td>
</tr>
<tr>
<td>MQCSS</td>
<td>3139.76</td>
<td>0.2360</td>
<td>5</td>
<td>148.20</td>
<td>LQCSS</td>
</tr>
<tr>
<td>LQCSS</td>
<td>148.20</td>
<td>1.7100</td>
<td>5</td>
<td>50.68</td>
<td>LLOQCSS</td>
</tr>
</tbody>
</table>

**Spiked Calibration Curve Standards in plasma**

The above calibration curve dilutions (CC spiking solutions) were used to spike the screened blank human plasma matrix to prepare the plasma calibration curve standards ranging from 1.00 to 150.76 µg/mL as per the table given below.
### Spiked QC Plasma Samples

The above quality control dilutions (QC spiking solutions) were used to spike the screened blank human plasma to prepare the plasma quality control plasma samples ranging from 0.202 to 10.374 µg/mL as per the table given below.

<table>
<thead>
<tr>
<th>Stock/SS ID</th>
<th>Stock/SS Conc. (µg/mL)</th>
<th>Stock/SS Volume (mL)</th>
<th>Made up to with plasma (mL)</th>
<th>Final Conc. (µg/mL)</th>
<th>QC ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQCSS</td>
<td>5814.38</td>
<td>1.000</td>
<td>50</td>
<td>116.29</td>
<td>HQC</td>
</tr>
<tr>
<td>MQCSS</td>
<td>3139.76</td>
<td>0.5000</td>
<td>25</td>
<td>62.80</td>
<td>MQC</td>
</tr>
<tr>
<td>LQCSS</td>
<td>148.20</td>
<td>1.0000</td>
<td>50</td>
<td>2.96</td>
<td>LQC</td>
</tr>
<tr>
<td>LLOQQCSS</td>
<td>50.68</td>
<td>0.5000</td>
<td>25</td>
<td>1.01</td>
<td>LLOQQC</td>
</tr>
</tbody>
</table>

0.6 mL aliquots of the above plasma quality control samples were taken in pre labeled polypropylene vials which were then capped properly and stored in a freezer at –70°C.
B) METHOD DEVELOPMENT AND OPTIMIZATION OF THE CHROMATOGRAPHIC CONDITIONS

For developing the method for the assay of Felbamate, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. A non-polar Hypersil BDS C\textsubscript{18} column was chosen as the stationary phase for this study.

**The mobile phase and the flow rate**

In order to get sharp peaks and base line separation of the components, the author has carried out a number of experiments by varying the commonly used solvents, their compositions and flow rate.

To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases on a C\textsubscript{18} stationary phase. The buffer solution of 15mM Potassium di-hydrogen phosphate (3.6 ± 0.1) and acetonitrile in a ratio of 90:10 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing.

A mobile phase flow rate of 1.0 mL/min. was found to be suitable in the study range of 0.5 -1.5 mL/min.

**Detection wave length**

The UV absorption spectrum of the drug was taken in methanol and the $\lambda_{\text{max}}$ was found to be at 216 nm. Hence detection of the drug was monitored at 216 nm.
Retention time of Felbamate

A model chromatogram showing the separation of Felbamate was presented in Fig-5 under the above optimized conditions at retention time 11.9 and 4.8 min were obtained for Felbamate and Zidovudine respectively (Furthe information pl.see. chromatograms).

Data acquisition and processing
The chromatograms were obtained and data were processed by the peak area ratio method using the Class VP Software. The concentrations of the unknown samples were calculated from the following equation of the regression analysis of the spiked plasma calibration graph using $1/X^2$ as weighting factor.

$$Y = m X + C$$

$X =$ Analyte concentration / Internal standard concentration
$Y =$ Analyte area / Internal standard area (area ratio)
$m =$ Slope of the calibration curve
$C =$ Y intercept value

### Optimized Chromatographic Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Hypersil, BDS C$_{18}$ (4.6 X 250 mm, 5µ)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>15mM Potassium Di–hydrogen Phosphate (3.6 ± 0.1) and Acetonitrile (90: 10 v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 mL/min</td>
</tr>
<tr>
<td>Column oven temperature</td>
<td>35 ± 2°C</td>
</tr>
<tr>
<td>Auto sampler temperature</td>
<td>10°C</td>
</tr>
<tr>
<td>Volume of injection</td>
<td>20 µL</td>
</tr>
<tr>
<td>Run time</td>
<td>15.0 minutes</td>
</tr>
<tr>
<td>Detection wave length</td>
<td>216 nm</td>
</tr>
<tr>
<td>Retention time of Felbamate</td>
<td>11.9 minutes.</td>
</tr>
<tr>
<td>Retention time of Zidovudine</td>
<td>4.8 minutes.</td>
</tr>
</tbody>
</table>
Extraction process of plasma samples and their drying

Five hundred micro liters of the spiked plasma calibration curve standards and the quality control samples were transferred to a set of pre-labeled polypropylene tubes containing 50 µL of Zidovudine dilution (internal standard; 50 µg/mL). The tubes were vortexed for ten seconds. Each of the HLB 30mg/1CC cartridges was conditioned with 1mL of methanol followed by equilibrating with 1mL of 0.5% ortho phosphoric acid in water on the solid phase extraction chamber. The above samples were loaded on to the cartridges and the cartridges were again washed with 1mL of 0.5 % ortho phosphoric acid in water followed by 1mL of 10% methanol in water. The cartridges were dried for about one minute and eluted with 1mL of 1% ammonia in methanol. The eluents were evaporated in a stream of nitrogen for 20 min at 40°C and the residues in the dried tubes were reconstituted with 0.4 mL of the mobile phase. The contents of the tubes were vortexed and transferred into auto-sampler vials and then analyzed by HPLC. An aliquot of 20 µL of the sample was drawn each time from the vials in the auto sampler.

Based on chromatograms in Fig-2 represented no interference at anlayte and ISTD from the extracted blank plasma sample, Fig-3 shows there is no interference in the presence of extracted blank + ISTD plasma sample, Fig-4 represented extracted STD-1 (LLOQ) plasma sample and Fig-5 showed the extracted STD-8 (ULOQ) plasma sample of Felbamate (Further information pl. see. chromatograms).
C) METHOD VALIDATION PARAMETERS

i) Auto sampler carry over test (ASCOT)

The ASCOT was determined by sequentially injecting the aqueous mobile phase-1, reference solution (Drug and ISTD), mobile phase-2 as well as extracted blank plasma sample, the extracted ULOQ standard and once again the extracted blank plasma sample and calculating the percentage of the interference in analyte and the internal standard carried over to the latter aqueous and blank plasma sample.

Table-1
Percent carryover of the drug and the internal standard

<table>
<thead>
<tr>
<th>Auto sampler carry over test (ASCOT)</th>
<th>% Carryover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Name</td>
<td>Analyte area</td>
</tr>
<tr>
<td>Mobile phase-1</td>
<td>164</td>
</tr>
<tr>
<td>Reference Solution</td>
<td>1507489</td>
</tr>
<tr>
<td>Mobile phase-2</td>
<td>127</td>
</tr>
<tr>
<td>Extracted Plasma Blank-1</td>
<td>146</td>
</tr>
<tr>
<td>Extracted ULOQ</td>
<td>1041257</td>
</tr>
<tr>
<td>Extracted Plasma Blank-2</td>
<td>186</td>
</tr>
</tbody>
</table>

Percent carryover of the analyte = \( \frac{(127 - 164)}{1507489} \times 100 = 0.0 \%

ii) Screening of plasma lots and specificity

The selectivity of the present method was evaluated by checking the blank plasma containing with anticoagulant dipotassium ethylene dimaine tetraacetic acid (EDTA) obtained from different blood donors. Six different lots of blank plasma were screened and all of them were found to have no significant endogenous interferences at the retention times of the analyte and the internal standard. The same human EDTA plasma
lots free of interfering substances were used to prepare the calibration curve standards and the quality control samples for the validation study.

Table-2: Percent interferences at the retention times of the drug and the internal standards

<table>
<thead>
<tr>
<th>Plasma Lot No.</th>
<th>Area at Analyte RT in blank</th>
<th>Analyte Area in LLOQ</th>
<th>Interference Area (%) at analyte RT</th>
<th>IS Area In Blank</th>
<th>IS Area In LLOQ</th>
<th>Interference Area (%) at IS RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank-10</td>
<td>154</td>
<td>6452</td>
<td>2.4</td>
<td>50</td>
<td>425781</td>
<td>0.0</td>
</tr>
<tr>
<td>Blank-11</td>
<td>102</td>
<td>6118</td>
<td>1.6</td>
<td>211</td>
<td>455417</td>
<td>0.0</td>
</tr>
<tr>
<td>Blank-12</td>
<td>98</td>
<td>6398</td>
<td>1.5</td>
<td>154</td>
<td>419693</td>
<td>0.0</td>
</tr>
<tr>
<td>Blank-13</td>
<td>126</td>
<td>6704</td>
<td>2.0</td>
<td>144</td>
<td>466322</td>
<td>0.0</td>
</tr>
<tr>
<td>Blank-14</td>
<td>57</td>
<td>6619</td>
<td>0.9</td>
<td>169</td>
<td>460031</td>
<td>0.0</td>
</tr>
<tr>
<td>Blank-15</td>
<td>154</td>
<td>6452</td>
<td>2.4</td>
<td>109</td>
<td>461205</td>
<td>0.0</td>
</tr>
<tr>
<td>Mean</td>
<td>6423.8</td>
<td></td>
<td></td>
<td></td>
<td>448074.8</td>
<td></td>
</tr>
</tbody>
</table>

iii) Linearity

The linearity of the method was determined by a weighting factor \(1/X^2\) where \(X\) is concentration) least square regression analysis of the standard plots associated with the eight point standard curve for Felbamate. The calibration line was linear in the range of 1.00 to 150.76 µg/mL of the drug as shown in Fig-5. A straight-line fit made through the data points by least square regression analysis showed a constant proportionality with minimal data scattering. The correlation coefficient \((r)\) ranged from 0.9965 to 0.9995 for Felbamate.
Table-3: Back calculated concentrations of Felbamate and calibration curve parameters

<table>
<thead>
<tr>
<th>STD ID</th>
<th>Conc. (µg/mL)</th>
<th>FEL STD1</th>
<th>FEL STD2</th>
<th>FEL STD3</th>
<th>FEL STD4</th>
<th>FEL STD5</th>
<th>FEL STD6</th>
<th>FEL STD7</th>
<th>FEL STD8</th>
<th>Slope (m)</th>
<th>Intercept (c)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEL CC1</td>
<td>1.00</td>
<td>2.00</td>
<td>8.05</td>
<td>15.35</td>
<td>40.40</td>
<td>80.81</td>
<td>120.61</td>
<td>150.76</td>
<td></td>
<td>0.0186</td>
<td>-0.0293</td>
<td>0.9965</td>
</tr>
<tr>
<td>FEL CC2</td>
<td>0.94</td>
<td>2.18</td>
<td>8.16</td>
<td>14.28</td>
<td>40.57</td>
<td>80.44</td>
<td>117.69</td>
<td>153.52</td>
<td></td>
<td>0.0174</td>
<td>-0.0047</td>
<td>0.9995</td>
</tr>
<tr>
<td>FEL CC3</td>
<td>1.04</td>
<td>1.89</td>
<td>8.15</td>
<td>16.31</td>
<td>40.20</td>
<td>67.56*</td>
<td>121.37</td>
<td>147.82</td>
<td></td>
<td>0.0179</td>
<td>-0.0195</td>
<td>0.9958</td>
</tr>
<tr>
<td>FEL CC4</td>
<td>1.12</td>
<td>1.98</td>
<td>7.58</td>
<td>15.80</td>
<td>42.35</td>
<td>78.60</td>
<td>122.92</td>
<td>152.94</td>
<td></td>
<td>0.0184</td>
<td>-0.0084</td>
<td>0.9993</td>
</tr>
<tr>
<td>FEL CC5</td>
<td>0.97</td>
<td>1.86</td>
<td>8.41</td>
<td>12.30*</td>
<td>39.74</td>
<td>81.69</td>
<td>122.03</td>
<td>151.22</td>
<td></td>
<td>0.0196</td>
<td>-0.0134</td>
<td>0.9995</td>
</tr>
<tr>
<td>FEL CC6</td>
<td>1.03</td>
<td>2.21</td>
<td>7.59</td>
<td>15.64</td>
<td>39.01</td>
<td>77.60</td>
<td>121.46</td>
<td>149.85</td>
<td></td>
<td>0.0164</td>
<td>-0.0275</td>
<td>0.9978</td>
</tr>
</tbody>
</table>

Mean 1.022 2.010 8.000 14.893 39.968 77.715 120.205 151.210
±SD 0.0624 0.1494 0.3385 1.4496 1.4948 5.1837 2.8184 2.1053
%CV 6.1 7.4 4.2 9.7 3.7 6.7 2.3 1.4
%Nominal 102.2 100.5 99.4 97.0 98.9 96.2 99.7 100.3

* Indicates out of acceptance limits.

Calibration curve was drawn by using nominal concentration and area ratio of calibration curve (FEL CC1); the values were given below in table-4.
Table-4: Nominal concentrations and area ratio of Felbamate.

<table>
<thead>
<tr>
<th>Nominal Conc.</th>
<th>Area Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0.0183</td>
</tr>
<tr>
<td>2.00</td>
<td>0.0341</td>
</tr>
<tr>
<td>8.05</td>
<td>0.1447</td>
</tr>
<tr>
<td>15.35</td>
<td>0.2719</td>
</tr>
<tr>
<td>40.40</td>
<td>0.6817</td>
</tr>
<tr>
<td>80.81</td>
<td>1.3993</td>
</tr>
<tr>
<td>120.61</td>
<td>2.0637</td>
</tr>
<tr>
<td>150.76</td>
<td>2.9351</td>
</tr>
</tbody>
</table>

iv) Precision and Accuracy

The precision of the assay was calculated by the percent coefficient of variation for QC samples of Felbamate. The accuracy of the assay was calculated by computing the ratio of the calculated mean values of the QC samples to their respective nominal values, expressed as percentage nominal.

Within-batch Precision for Felbamate (% CV)
Within-batch precision for LLOQ QC ranged from 5.7 to 8.5%
Within-batch precision for LQC, MQC and HQC ranged from 1.9 to 7.5%

Between-batch Precision for Felbamate (% CV)
Between-batch precision for LLOQ QC was 8.0%
Between-batch precision for LQC, MQC and HQC ranged from 5.2 to 7.1%

Within-batch Accuracy for Felbamate (% Nominal)
Within-batch accuracy for LLOQ QC ranged from 93.6 to 104.6%
Within-batch accuracy for LQC, MQC and HQC ranged from 91.2 to 109.4%
Between-batch Accuracy for Felbamate (% Nominal)
Between-batch accuracy for LLOQ QC was 99.3%
Between-batch accuracy for LQC, MQC and HQC ranged from 99.8 to 105.1%

Table-5: Back calculated concentration of QC samples for Felbamate (within-batch)

<table>
<thead>
<tr>
<th>BATCH ID</th>
<th>QC ID</th>
<th>FEL LLOQQC</th>
<th>FEL LQC</th>
<th>FEL MQC</th>
<th>FEL HQC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Conc. (µg/mL)</td>
<td>1.01</td>
<td>2.96</td>
<td>62.80</td>
<td>116.29</td>
<td></td>
</tr>
<tr>
<td>PA 01</td>
<td>1</td>
<td>1.09</td>
<td>2.89</td>
<td>60.41</td>
<td>125.81</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.17</td>
<td>2.94</td>
<td>62.89</td>
<td>129.64</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.94</td>
<td>2.77</td>
<td>60.36</td>
<td>130.80</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.03</td>
<td>2.95</td>
<td>66.27</td>
<td>125.41</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.08</td>
<td>3.05</td>
<td>68.42</td>
<td>124.80</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.98</td>
<td>3.19</td>
<td>63.90</td>
<td>126.73</td>
</tr>
<tr>
<td>Mean</td>
<td>1.048</td>
<td>2.965</td>
<td>63.708</td>
<td>127.198</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.0828</td>
<td>0.1431</td>
<td>3.2112</td>
<td>2.4507</td>
<td></td>
</tr>
<tr>
<td>%CV</td>
<td>7.9</td>
<td>4.8</td>
<td>5.0</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>%Nominal</td>
<td>103.8</td>
<td>100.2</td>
<td>101.4</td>
<td>109.4</td>
<td></td>
</tr>
<tr>
<td>PA 02</td>
<td>7</td>
<td>1.10</td>
<td>2.88</td>
<td>64.41</td>
<td>120.36</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.04</td>
<td>2.79</td>
<td>66.79</td>
<td>122.81</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.92</td>
<td>2.80</td>
<td>73.61</td>
<td>125.94</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.05</td>
<td>2.94</td>
<td>68.91</td>
<td>129.36</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.91</td>
<td>3.07</td>
<td>64.70</td>
<td>130.58</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.03</td>
<td>3.11</td>
<td>69.42</td>
<td>123.65</td>
</tr>
<tr>
<td>Mean</td>
<td>1.008</td>
<td>2.932</td>
<td>67.973</td>
<td>125.450</td>
<td></td>
</tr>
<tr>
<td>±SD</td>
<td>0.0763</td>
<td>0.1350</td>
<td>3.4512</td>
<td>3.9487</td>
<td></td>
</tr>
<tr>
<td>%CV</td>
<td>7.6</td>
<td>4.6</td>
<td>5.1</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>%Nominal</td>
<td>99.8</td>
<td>99.0</td>
<td>108.2</td>
<td>107.9</td>
<td></td>
</tr>
<tr>
<td>PA 03</td>
<td>13</td>
<td>1.04</td>
<td>3.04</td>
<td>63.21</td>
<td>121.86</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1.00</td>
<td>3.15</td>
<td>66.93</td>
<td>125.37</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.93</td>
<td>3.11</td>
<td>61.85</td>
<td>120.84</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.86</td>
<td>3.18</td>
<td>65.90</td>
<td>126.28</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.97</td>
<td>3.07</td>
<td>67.59</td>
<td>128.06</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.92</td>
<td>2.95</td>
<td>66.74</td>
<td>122.73</td>
</tr>
<tr>
<td>Mean</td>
<td>0.953</td>
<td>3.083</td>
<td>65.370</td>
<td>124.190</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.0638</td>
<td>0.0829</td>
<td>2.3055</td>
<td>2.8114</td>
<td></td>
</tr>
<tr>
<td>%CV</td>
<td>6.7</td>
<td>2.7</td>
<td>3.5</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>%Nominal</td>
<td>94.4</td>
<td>104.2</td>
<td>104.1</td>
<td>106.8</td>
<td></td>
</tr>
<tr>
<td>PA 04</td>
<td>19</td>
<td>0.92</td>
<td>2.87</td>
<td>59.82</td>
<td>120.48</td>
</tr>
<tr>
<td></td>
<td>20</td>
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<td>2.96</td>
<td>57.96</td>
<td>124.57</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0.89</td>
<td>2.41</td>
<td>59.18</td>
<td>126.31</td>
</tr>
</tbody>
</table>
### Table-6: Back calculated concentration of QC Samples for Felbamate (between-batches)

<table>
<thead>
<tr>
<th>QC ID</th>
<th>FEL LLOQQC</th>
<th>FEL LQC</th>
<th>FEL MQC</th>
<th>FEL RHQC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Conc. (µg/mL)</td>
<td>1.01</td>
<td>2.96</td>
<td>62.80</td>
<td>116.29</td>
</tr>
<tr>
<td>Mean</td>
<td>1.008</td>
<td>2.953</td>
<td>62.666</td>
<td>122.261</td>
</tr>
<tr>
<td>±SD</td>
<td>0.0804</td>
<td>0.1526</td>
<td>4.4487</td>
<td>7.6009</td>
</tr>
<tr>
<td>%CV</td>
<td>8.0</td>
<td>5.2</td>
<td>7.1</td>
<td>6.2</td>
</tr>
<tr>
<td>%Nominal</td>
<td>99.8</td>
<td>99.8</td>
<td>99.8</td>
<td>105.1</td>
</tr>
</tbody>
</table>
v) Dilution Integrity

A quality control pool (containing 301.52 µg/mL of Felbamate) was prepared in plasma EDTA at a concentration of approximately twice the high CC standard (ULOQ) to assess the dilution integrity. The precision and accuracy for dilution integrity at 50% dilution and 25% dilution of the QC pool sample with screened blank human plasma were determined by using calibration curve standards. The precision for dilution integrity of Felbamate was 2.6% at 25 percent dilution and 1.4% at 50 percent dilution. The accuracy for dilution integrity of Felbamate was 95.4% for 25 percent dilution and 96.8% for 50 percent dilution. Results were shown in the following table.

Table-7: Back calculated concentrations for CC standards of Felbamate

<table>
<thead>
<tr>
<th>STD ID</th>
<th>FEL STD1</th>
<th>FEL STD2</th>
<th>FEL STD3</th>
<th>FEL STD4</th>
<th>FEL STD5</th>
<th>FEL STD6</th>
<th>FEL STD7</th>
<th>FEL STD8</th>
<th>Slope (m)</th>
<th>Intercept (c)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc. (µg/mL)</td>
<td>1.00</td>
<td>2.00</td>
<td>8.05</td>
<td>15.35</td>
<td>40.40</td>
<td>80.81</td>
<td>120.61</td>
<td>150.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEL CC7</td>
<td>1.01</td>
<td>1.92</td>
<td>8.27</td>
<td>16.34</td>
<td>37.39</td>
<td>84.66</td>
<td>122.30</td>
<td>153.61</td>
<td>0.0194</td>
<td>0.0030</td>
<td>0.9994</td>
</tr>
<tr>
<td>%Nominal</td>
<td>101.0</td>
<td>96.0</td>
<td>102.7</td>
<td>106.4</td>
<td>92.5</td>
<td>104.8</td>
<td>101.4</td>
<td>101.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table-8: Back calculated concentrations for quality control dilution samples in plasma

<table>
<thead>
<tr>
<th>Nominal Concentration</th>
<th>Felbamate dilution integrity quality control samples (~2 Times Conc. of ULOQC 301.52 µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution factor</td>
<td>50% dilution</td>
</tr>
<tr>
<td>Batch ID</td>
<td>FEL DIQC DF 2</td>
</tr>
<tr>
<td>S. No.</td>
<td>Obtained Conc. (µg/mL)</td>
</tr>
<tr>
<td></td>
<td>Obtained Conc. (µg/mL)</td>
</tr>
<tr>
<td>1</td>
<td>294.85</td>
</tr>
<tr>
<td>2</td>
<td>296.32</td>
</tr>
<tr>
<td>3</td>
<td>290.41</td>
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<tr>
<td>4</td>
<td>284.63</td>
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<tr>
<td>5</td>
<td>293.64</td>
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<tr>
<td>6</td>
<td>291.97</td>
</tr>
<tr>
<td>Mean</td>
<td>291.970</td>
</tr>
<tr>
<td>±SD</td>
<td>4.1549</td>
</tr>
<tr>
<td>%CV</td>
<td>1.4</td>
</tr>
<tr>
<td>% Nominal</td>
<td>96.8</td>
</tr>
</tbody>
</table>
vi) Recovery

a) Recovery of the drug

The percent recoveries were determined by comparing the areas of the extracted QC samples with equivalent aqueous samples. Recovery for Felbamate ranges from 67.3% to 71.8% (Mean Recovery: 69.32%).

Table-9: Recovery of Felbamate in human plasma

<table>
<thead>
<tr>
<th>QC ID</th>
<th>Un Extracted</th>
<th>Extracted</th>
<th>Un Extracted</th>
<th>Extracted</th>
<th>Un Extracted</th>
<th>Extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEL AQ RLQC 1-6</td>
<td>FEL LQC</td>
<td>FEL AQ RMQC 1-6</td>
<td>FEL MQC</td>
<td>FEL AQ RHQC 1-6</td>
<td>FEL HQC</td>
</tr>
<tr>
<td>37</td>
<td>28410</td>
<td>20148</td>
<td>621437</td>
<td>411028</td>
<td>1152473</td>
<td>798410</td>
</tr>
<tr>
<td>38</td>
<td>29753</td>
<td>19981</td>
<td>610741</td>
<td>402783</td>
<td>1054007</td>
<td>772851</td>
</tr>
<tr>
<td>39</td>
<td>30138</td>
<td>21653</td>
<td>607192</td>
<td>421549</td>
<td>1131475</td>
<td>770064</td>
</tr>
<tr>
<td>40</td>
<td>28963</td>
<td>21014</td>
<td>610036</td>
<td>415749</td>
<td>1100383</td>
<td>792483</td>
</tr>
<tr>
<td>41</td>
<td>27450</td>
<td>20143</td>
<td>609410</td>
<td>402743</td>
<td>1241740</td>
<td>801426</td>
</tr>
<tr>
<td>42</td>
<td>29852</td>
<td>22316</td>
<td>611424</td>
<td>415729</td>
<td>1198</td>
<td>805769</td>
</tr>
<tr>
<td>Mean</td>
<td>29094.3</td>
<td>20875.8</td>
<td>611706.7</td>
<td>411596.8</td>
<td>1146462.2</td>
<td>790167.2</td>
</tr>
<tr>
<td>±SD</td>
<td>1028.25</td>
<td>955.48</td>
<td>4982.77</td>
<td>7612.60</td>
<td>67436.08</td>
<td>15149.08</td>
</tr>
<tr>
<td>%CV</td>
<td>3.5</td>
<td>4.6</td>
<td>0.8</td>
<td>1.8</td>
<td>5.9</td>
<td>1.9</td>
</tr>
<tr>
<td>% Recovery</td>
<td>71.8</td>
<td>67.3</td>
<td>68.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table-10: Global recovery of Felbamate

<table>
<thead>
<tr>
<th>QC Level</th>
<th>LQC</th>
<th>MQC</th>
<th>HQC</th>
<th>Mean</th>
<th>±SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Recovery</td>
<td>71.8</td>
<td>67.3</td>
<td>68.9</td>
<td>69.32</td>
<td>2.259</td>
<td>3.3</td>
</tr>
</tbody>
</table>

b) Recovery of the internal standard
The percent recovery of the IS (Zidovudine) was determined by comparing the areas of the extracted IS samples with equivalent aqueous IS samples at MQC level. The recovery obtained for Zidovudine was 93.3%.

Table-11: Recovery of Zidovudine (Internal standard)

<table>
<thead>
<tr>
<th>QC ID</th>
<th>Un Extracted</th>
<th>Extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEL AQ RMQC</td>
<td>FEL MQC</td>
</tr>
<tr>
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<td>689180</td>
<td>402178</td>
</tr>
<tr>
<td>38</td>
<td>702236</td>
<td>395746</td>
</tr>
<tr>
<td>39</td>
<td>671418</td>
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<td>715624</td>
<td>354983</td>
</tr>
<tr>
<td>41</td>
<td>700812</td>
<td>398741</td>
</tr>
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<td>698756</td>
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<tr>
<td>Mean</td>
<td>696337.7</td>
<td>389095.5</td>
</tr>
<tr>
<td>±SD</td>
<td>14865.01</td>
<td>17909.07</td>
</tr>
<tr>
<td>%CV</td>
<td>2.1</td>
<td>4.6</td>
</tr>
</tbody>
</table>

% Recovery 55.9

vii) STABILITY

A) STABILITY OF THE DRUGS IN STOCK SOLUTION

i) Short-term stability of the drugs in stock solution

Stock solutions of about 5mg/mL of Felbamate and Zidovudine (IS) were prepared freshly in Methanol and water (60:40) and a portion of the freshly prepared stock solution (Stability samples) was kept at room temperature of ~25°C for 8.35 hrs. The remaining portions of the above stock solutions were left in refrigerator below 10°C, which were used as comparison samples.

The Short-term stock solution stability of the Felbamate and Zidovudine in methanol and water (60:40) was assessed by comparing the mean of the responses of six replicates of the stability samples with that of the six replicates of the comparison samples. After keeping for 8.35 hrs at a room temperature of ~ 25°C, the percent stabilities were found to be 98.9 and 97.9 for Felbamate and Zidovudine respectively.
Table-12: Short-term stability of Felbamate & Zidovudine in stock solutions

<table>
<thead>
<tr>
<th>Replicate No.</th>
<th>Felbamate</th>
<th>Zidovudine (IS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Comparison (0.0 hour)</td>
<td>Stability (8.35 hours)</td>
</tr>
<tr>
<td>1</td>
<td>1642806</td>
<td>1620057</td>
</tr>
<tr>
<td>2</td>
<td>1698357</td>
<td>1639582</td>
</tr>
<tr>
<td>3</td>
<td>1742210</td>
<td>1601498</td>
</tr>
<tr>
<td>4</td>
<td>1694351</td>
<td>1854732</td>
</tr>
<tr>
<td>5</td>
<td>1659839</td>
<td>1753671</td>
</tr>
<tr>
<td>6</td>
<td>1744266</td>
<td>1601587</td>
</tr>
<tr>
<td>Mean</td>
<td>1696972</td>
<td>1678521</td>
</tr>
<tr>
<td>±SD</td>
<td>41484</td>
<td>103425</td>
</tr>
<tr>
<td>%CV</td>
<td>2.4</td>
<td>6.2</td>
</tr>
<tr>
<td>% Stability</td>
<td>98.9</td>
<td>97.9</td>
</tr>
</tbody>
</table>

ii) Long-term stability of drugs in stock solution

Stock solutions of about 5 mg/mL of Felbamate and Zidovudine were prepared freshly in methanol and water (60:40) and a portion of the stock solution was stored in a refrigerator below 10°C (Stability samples) for 14.6 days. On the day of long term stability analysis, the stock solutions were freshly prepared and used as comparison samples.

The long-term stock solution stability of Felbamate and Zidovudine in methanol and water (60:40) was assessed by comparing the mean of the responses of six replicates of the stability samples with that of the six replicates of the comparison samples. After keeping for 14.6 days in a refrigerator below 10°C, the percent stabilities obtained were 102.0 and 101.2 for Felbamate and Zidovudine respectively.
Table-13: Long-term stability of Felbamate and Zidovudine in stock solution

<table>
<thead>
<tr>
<th>Replicate No.</th>
<th>Felbamate</th>
<th>Zidovudine (IS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Comparison (0.0 hour)</td>
<td>Stability (14.6 Days)</td>
</tr>
<tr>
<td>1</td>
<td>1562870</td>
<td>1632589</td>
</tr>
<tr>
<td>2</td>
<td>1526981</td>
<td>1569357</td>
</tr>
<tr>
<td>3</td>
<td>1624953</td>
<td>1562954</td>
</tr>
<tr>
<td>4</td>
<td>1502479</td>
<td>1548926</td>
</tr>
<tr>
<td>5</td>
<td>1566285</td>
<td>1562951</td>
</tr>
<tr>
<td>6</td>
<td>1560027</td>
<td>1649785</td>
</tr>
<tr>
<td>Mean</td>
<td>1557232.5</td>
<td>1587760.3</td>
</tr>
<tr>
<td>±SD</td>
<td>41457.58</td>
<td>42271.30</td>
</tr>
<tr>
<td>%CV</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>% Stability</td>
<td>102.0</td>
<td></td>
</tr>
</tbody>
</table>

B) STABILITY OF DRUGS IN BIOLOGICAL MATRIX

In-injector stability or wet extract stability

In-injector stability of Felbamate in plasma was determined by storing the processed QC samples (Stability samples) for 24.00 hrs at 10°C in the auto sampler and then analyzing them along with six replicates of freshly prepared quality control samples (Comparison samples) at low and high concentration levels (known from a freshly prepared calibration curve). The percent In-injector stability was calculated from the mean of the concentrations of stability samples stored at 10°C for 24.00 hrs in the auto sampler and the mean of the comparison samples.

The In-injector stability values of Felbamate in human plasma after 24.00 hrs were 92.7% and 98.3% at low and high concentrations respectively.
Table-14: Back calculated concentrations for CC standards of Felbamate

<table>
<thead>
<tr>
<th>STD ID</th>
<th>FEL STD1</th>
<th>FEL STD2</th>
<th>FEL STD3</th>
<th>FEL STD4</th>
<th>FEL STD5</th>
<th>FEL STD6</th>
<th>FEL STD7</th>
<th>FEL STD8</th>
<th>Slope (m)</th>
<th>Intercept (c)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc. (µg/mL)</td>
<td>1.00</td>
<td>2.00</td>
<td>8.05</td>
<td>15.35</td>
<td>40.40</td>
<td>80.81</td>
<td>120.61</td>
<td>150.76</td>
<td>0.0150</td>
<td>-0.0008</td>
<td>0.9993</td>
</tr>
<tr>
<td>FEL (FS) CC1</td>
<td>1.02</td>
<td>2.10</td>
<td>7.59</td>
<td>17.91</td>
<td>41.85</td>
<td>83.66</td>
<td>122.41</td>
<td>155.36</td>
<td>0.0150</td>
<td>-0.0008</td>
<td>0.9993</td>
</tr>
<tr>
<td>%Nominal</td>
<td>102.0</td>
<td>105.0</td>
<td>94.3</td>
<td>116.7</td>
<td>103.6</td>
<td>101.5</td>
<td>103.5</td>
<td>103.1</td>
<td>0.0150</td>
<td>-0.0008</td>
<td>0.9993</td>
</tr>
</tbody>
</table>

Table-15: In-injector stability of Felbamate (24.00 hrs)

<table>
<thead>
<tr>
<th>Felbamate</th>
<th>In-injector stability</th>
<th>Comparison Samples</th>
<th>Stability Samples</th>
<th>Comparison Samples</th>
<th>Stability Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Conc. (µg/mL)</td>
<td>2.96</td>
<td>2.96</td>
<td>116.29</td>
<td>116.29</td>
<td></td>
</tr>
<tr>
<td>QC ID</td>
<td>FEL (FS) LQC</td>
<td>QC ID</td>
<td>FEL LQC</td>
<td>QC ID</td>
<td>FEL (FS) HQC</td>
</tr>
<tr>
<td>1</td>
<td>3.02</td>
<td>49</td>
<td>2.84</td>
<td>1</td>
<td>126.91</td>
</tr>
<tr>
<td>2</td>
<td>3.14</td>
<td>50</td>
<td>2.93</td>
<td>2</td>
<td>123.58</td>
</tr>
<tr>
<td>3</td>
<td>3.21</td>
<td>51</td>
<td>2.99</td>
<td>3</td>
<td>122.07</td>
</tr>
<tr>
<td>4</td>
<td>3.07</td>
<td>52</td>
<td>2.48</td>
<td>4</td>
<td>129.44</td>
</tr>
<tr>
<td>5</td>
<td>2.95</td>
<td>53</td>
<td>2.91</td>
<td>5</td>
<td>123.37</td>
</tr>
<tr>
<td>6</td>
<td>2.83</td>
<td>54</td>
<td>2.74</td>
<td>6</td>
<td>120.81</td>
</tr>
<tr>
<td>Mean</td>
<td>3.037</td>
<td>2.815</td>
<td>124.363</td>
<td>122.248</td>
<td></td>
</tr>
<tr>
<td>±SD</td>
<td>0.1359</td>
<td>0.1851</td>
<td>3.2170</td>
<td>4.4124</td>
<td></td>
</tr>
<tr>
<td>%CV</td>
<td>4.5</td>
<td>6.6</td>
<td>2.6</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>%Nominal</td>
<td>102.6</td>
<td>95.1</td>
<td>106.9</td>
<td>105.1</td>
<td></td>
</tr>
<tr>
<td>% Stability</td>
<td>92.7</td>
<td>98.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Indicates out of acceptance limits.

Dry Extract stability

The stability of the extracted and dried samples of Felbamate from plasma (without reconstitution), was assessed by storing six replicates of the dried stability samples at a temperature below 10°C for 37.60 hrs and then analyzing after appropriate reconstitution along with six replicates of freshly prepared quality control samples (Comparison samples) at low and high concentration levels (known from a freshly prepared calibration curve). The percent stability was determined by using the mean of the concentrations of the QC samples stored below 10°C for 37.60 hrs and that of the comparison samples.
The dry extract stability values of Felbamate at a temperature below 10°C for 37.60 hrs were 95.6% and 94.8% at low and high concentrations respectively.

Table-16: Back calculated concentrations for CC standards of Felbamate

<table>
<thead>
<tr>
<th>STD ID</th>
<th>FEL STD1</th>
<th>FEL STD2</th>
<th>FEL STD3</th>
<th>FEL STD4</th>
<th>FEL STD5</th>
<th>FEL STD6</th>
<th>FEL STD7</th>
<th>FEL STD8</th>
<th>Slope (m)</th>
<th>Intercept (c)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc. (µg/mL)</td>
<td>1.00</td>
<td>2.00</td>
<td>8.05</td>
<td>15.35</td>
<td>40.40</td>
<td>80.81</td>
<td>120.61</td>
<td>150.76</td>
<td>0.0150</td>
<td>-0.0008</td>
<td>0.9993</td>
</tr>
<tr>
<td>FEL (FS) CC1</td>
<td>1.02</td>
<td>2.10</td>
<td>7.59</td>
<td>17.91</td>
<td>41.85</td>
<td>83.66</td>
<td>122.41</td>
<td>155.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Nominal</td>
<td>102.0</td>
<td>105.0</td>
<td>94.3</td>
<td>116.7</td>
<td>103.6</td>
<td>103.5</td>
<td>101.5</td>
<td>103.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table-17: Dry extract stability of Felbamate (37.60 hrs)

<table>
<thead>
<tr>
<th>Felbamate</th>
<th>Comparison Samples</th>
<th>Stability Samples</th>
<th>Comparison Samples</th>
<th>Stability Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Conc. (µg/mL)</td>
<td>2.96</td>
<td>2.96</td>
<td>116.29</td>
<td>116.29</td>
</tr>
<tr>
<td>QC ID</td>
<td>FEL (FS) LQC</td>
<td>QC ID</td>
<td>FEL LQC</td>
<td>QC ID</td>
</tr>
<tr>
<td>1</td>
<td>3.02</td>
<td>55</td>
<td>2.74</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>3.14</td>
<td>56</td>
<td>2.91</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>3.21</td>
<td>57</td>
<td>3.14</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>3.07</td>
<td>58</td>
<td>2.87</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>2.95</td>
<td>59</td>
<td>2.75</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>2.83</td>
<td>60</td>
<td>3.01</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td>3.037</td>
<td>2.903</td>
<td>124.363</td>
<td>117.937</td>
</tr>
<tr>
<td>±SD</td>
<td>0.1359</td>
<td>0.1541</td>
<td>3.2170</td>
<td>4.0799</td>
</tr>
<tr>
<td>%CV</td>
<td>4.5</td>
<td>5.3</td>
<td>2.6</td>
<td>3.5</td>
</tr>
<tr>
<td>%Nominal</td>
<td>102.6</td>
<td>98.1</td>
<td>106.9</td>
<td>101.4</td>
</tr>
<tr>
<td>% Stability</td>
<td>95.6</td>
<td>94.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Freeze-thaw stability

The Freeze-thaw stability of Felbamate in human plasma was determined by analyzing six replicates of the quality control samples at low and high concentration levels (Stability samples), previously frozen and thawed over four cycles along with six replicates of the freshly spiked (FS) quality control samples (Comparison samples) at low and high concentration levels (known from a freshly prepared calibration curve). The percent stability at low and high quality control concentration levels was calculated
by comparing the mean of the concentrations of stability samples with that of the comparison samples.

The Freeze-thaw stability values of Felbamate in plasma after four FT cycles were 97.9% and 96.6% at low and high concentrations respectively.

**Table-18: Back calculated concentrations for CC Standards of Felbamate (4 cycles)**

<table>
<thead>
<tr>
<th>STD ID</th>
<th>FEL STD1</th>
<th>FEL STD2</th>
<th>FEL STD3</th>
<th>FEL STD4</th>
<th>FEL STD5</th>
<th>FEL STD6</th>
<th>FEL STD7</th>
<th>FEL STD8</th>
<th>Slope (m)</th>
<th>Intercept (c)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc. (µg/mL)</td>
<td>1.00</td>
<td>2.00</td>
<td>8.05</td>
<td>15.35</td>
<td>40.40</td>
<td>80.81</td>
<td>120.61</td>
<td>150.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEL (FS) CC2</td>
<td>0.96</td>
<td>1.92</td>
<td>6.63</td>
<td>16.20</td>
<td>43.65</td>
<td>76.52</td>
<td>124.83</td>
<td>151.64</td>
<td>0.0165</td>
<td>-0.0054</td>
<td>0.9993</td>
</tr>
<tr>
<td>%Nominal</td>
<td>96.0</td>
<td>96.0</td>
<td>82.4</td>
<td>105.5</td>
<td>108.0</td>
<td>94.7</td>
<td>103.5</td>
<td>100.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table-19: Freeze-thaw stability of Felbamate in plasma (4 cycles)**

<table>
<thead>
<tr>
<th>QC ID</th>
<th>FEL (FS) LQC</th>
<th>QC ID</th>
<th>FEL LQC</th>
<th>QC ID</th>
<th>FEL (FS) HQC</th>
<th>QC ID</th>
<th>FEL HQC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.12 43</td>
<td>2.89 1</td>
<td>121.94 43</td>
<td>110.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.08 44</td>
<td>2.73 2</td>
<td>125.67 44</td>
<td>116.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.92 45</td>
<td>2.81 3</td>
<td>120.72 45</td>
<td>108.85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.89 46</td>
<td>3.17 4</td>
<td>117.90 46</td>
<td>121.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.10 47</td>
<td>3.44 5</td>
<td>124.17 47</td>
<td>122.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3.14 48</td>
<td>2.82 6</td>
<td>125.01 48</td>
<td>129.94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.042 2.977</td>
<td>122.568 118.345</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>±SD</td>
<td>0.1082 0.2730</td>
<td>2.9581</td>
<td>7.9616</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%CV</td>
<td>3.6 9.2</td>
<td>2.4</td>
<td>6.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Nominal</td>
<td>102.8</td>
<td>100.6</td>
<td>105.4</td>
<td>101.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Stability</td>
<td>97.9</td>
<td>96.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Indicates out of acceptance limits.

**Bench top stability**

For determining the bench top stability of Felbamate in human plasma six replicates of unprocessed stability samples, which were maintained at a temperature of
~ 25°C for 9.00 hrs and six replicates of the freshly prepared quality control samples (Comparison samples) at low and high QC concentration levels (known from a freshly prepared calibration curve) were analyzed and the percent stability at low and high QC concentration levels was calculated by comparing the mean of the concentrations of the stability samples with that of the comparison samples.

The Bench top stability values of Felbamate in plasma after 9.00 hrs were 100.2% and 110.0% at low and high concentrations respectively.

**Table-20: Back calculated concentrations for CC standards of Felbamate**

<table>
<thead>
<tr>
<th>STD ID</th>
<th>FEL STD1</th>
<th>FEL STD2</th>
<th>FEL STD3</th>
<th>FEL STD4</th>
<th>FEL STD5</th>
<th>FEL STD6</th>
<th>FEL STD7</th>
<th>FEL STD8</th>
<th>Slope (m)</th>
<th>Intercept (c)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc. (µg/mL)</td>
<td>1.00</td>
<td>2.00</td>
<td>8.05</td>
<td>15.35</td>
<td>40.40</td>
<td>80.81</td>
<td>120.61</td>
<td>150.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEL (FS) CC2</td>
<td>0.96</td>
<td>1.92</td>
<td>6.63</td>
<td>16.20</td>
<td>43.65</td>
<td>76.52</td>
<td>124.83</td>
<td>151.64</td>
<td>0.0165</td>
<td>-0.0054</td>
<td>0.9993</td>
</tr>
<tr>
<td>%Nominal</td>
<td>96.0</td>
<td>96.0</td>
<td>82.4</td>
<td>105.5</td>
<td>108.0</td>
<td>94.7</td>
<td>103.5</td>
<td>100.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table-21: Bench top stability of Felbamate (9.00 hrs)**

<table>
<thead>
<tr>
<th>Felbam</th>
<th>Comparison Samples</th>
<th>Stability Samples</th>
<th>Comparison Samples</th>
<th>Stability Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Conc. (µg/mL)</td>
<td>2.96</td>
<td>2.96</td>
<td>116.29</td>
<td>116.29</td>
</tr>
<tr>
<td>QC ID</td>
<td>FEL (FS) LQC</td>
<td>QC ID</td>
<td>FEL LQC</td>
<td>QC ID</td>
</tr>
<tr>
<td>1</td>
<td>3.12</td>
<td>43</td>
<td>3.16</td>
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</tr>
<tr>
<td>2</td>
<td>3.08</td>
<td>44</td>
<td>2.98</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>2.92</td>
<td>45</td>
<td>2.79</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>2.89</td>
<td>46</td>
<td>3.10</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>3.10</td>
<td>47</td>
<td>3.04</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>3.14</td>
<td>48</td>
<td>3.11</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td>3.042</td>
<td>3.030</td>
<td>122.568</td>
<td>121.108</td>
</tr>
<tr>
<td>±SD</td>
<td>0.1082</td>
<td>0.1330</td>
<td>2.9581</td>
<td>3.2400</td>
</tr>
<tr>
<td>%CV</td>
<td>3.6</td>
<td>4.4</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>%Nominal</td>
<td>102.8</td>
<td>102.4</td>
<td>105.4</td>
<td>104.1</td>
</tr>
<tr>
<td>% Stability</td>
<td>99.6</td>
<td>98.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Long-term stability in plasma matrix**

For finding out the long-term stability of Felbamate in human plasma the stability samples were stored at a temperature of -20°C for 21.00 days. Then they were
analyzed along with six replicates of the freshly prepared quality control samples (Comparison samples) at low and high concentration levels. The low and high concentration levels were read from the calibration curve. The quality control samples and the calibration curve standards were prepared by spiking the freshly prepared drug dilutions in screened blank plasma. The percent stabilities at low and high QC concentration levels were calculated by using the mean of the concentrations of the stability samples and the mean of the comparison samples.

The long-term stability values obtained for Felbamate in human EDTA plasma at a temperature of -20°C for 21.00 days were 98.5% and 97.0% at low and high concentrations respectively.

Table-22: Back calculated concentrations for CC standards of Felbamate

<table>
<thead>
<tr>
<th>STD ID</th>
<th>STD1</th>
<th>STD2</th>
<th>STD3</th>
<th>STD4</th>
<th>STD5</th>
<th>STD6</th>
<th>STD7</th>
<th>STD8</th>
<th>Slope (m)</th>
<th>Intercept (c)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc. (µg/mL)</td>
<td>1.00</td>
<td>2.00</td>
<td>8.04</td>
<td>15.35</td>
<td>40.40</td>
<td>80.80</td>
<td>120.60</td>
<td>150.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEL FS2CC1</td>
<td>0.94</td>
<td>2.13</td>
<td>8.18</td>
<td>14.80</td>
<td>38.72</td>
<td>81.64</td>
<td>124.63</td>
<td>152.86</td>
<td>0.0171</td>
<td>0.0038</td>
<td>0.9991</td>
</tr>
<tr>
<td>%Nominal</td>
<td>94.0</td>
<td>106.5</td>
<td>101.7</td>
<td>96.4</td>
<td>95.8</td>
<td>101.0</td>
<td>103.3</td>
<td>101.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table-23: Long-term stability of Felbamate (21.00 days) at -20°C in EDTA

<table>
<thead>
<tr>
<th>Felbamate</th>
<th>Long-term stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Comparison Samples</td>
</tr>
<tr>
<td>Nominal Conc. (µg/mL)</td>
<td>2.96</td>
</tr>
<tr>
<td>QC ID</td>
<td>FELFS1 LQC</td>
</tr>
<tr>
<td>1</td>
<td>2.98</td>
</tr>
<tr>
<td>2</td>
<td>3.19</td>
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<tr>
<td>3</td>
<td>3.28</td>
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<tr>
<td>4</td>
<td>3.10</td>
</tr>
<tr>
<td>5</td>
<td>2.94</td>
</tr>
<tr>
<td>6</td>
<td>2.88</td>
</tr>
<tr>
<td>Mean</td>
<td>3.062</td>
</tr>
<tr>
<td>±SD</td>
<td>0.1550</td>
</tr>
<tr>
<td>%CV</td>
<td>5.1</td>
</tr>
<tr>
<td>%Nominal</td>
<td>103.4</td>
</tr>
<tr>
<td>Correction Factor</td>
<td>1.0000</td>
</tr>
<tr>
<td>Corrected Mean</td>
<td>3.015</td>
</tr>
<tr>
<td>% Stability</td>
<td>98.5</td>
</tr>
</tbody>
</table>

4) Summary of the results and conclusion
Table-24: summary of the results

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening of plasma lots and specificity</td>
<td>No significant interfering peaks were observed at the retention time of analyte (Felbamate) and Internal standard (IS; Zidovudine).</td>
</tr>
<tr>
<td>Calibration Curve</td>
<td>Calibration range: 1.00 to 150.76 µg/mL Accuracy: 96.2-102.2 % nominal Precision: 1.4 -9.7 %CV Correlation coefficient (r): 0.9958 - 0.9995</td>
</tr>
<tr>
<td>Precision (%CV)</td>
<td>Within-batch (LLOQ QC): 5.7 to 8.5 LQC, MQC &amp; HQC: 1.9 to 7.5 Between-batch (LLOQ QC): 8.0 LQC, MQC &amp; HQC: 5.2 to 7.1</td>
</tr>
<tr>
<td>Accuracy (% Nominal Conc.)</td>
<td>Within-batch (LLOQ QC): 93.6 to 104.6 L, M &amp; HQC: 91.2 to 109.4 Between-batch (LLOQ QC): 99.3 L, M &amp; HQC: 99.8 to 105.1</td>
</tr>
<tr>
<td>Dilution Integrity</td>
<td>At 50% dilution: Precision: 1.4 %CV Accuracy: 96.8 %Nominal At 25% dilution: Precision: 2.6 %CV Accuracy: 95.4 %Nominal</td>
</tr>
<tr>
<td>Recovery</td>
<td>Felbamate: Mean of % Recovery- 69.32% CV for % Recovery - 3.3 Zidovudine (IS): % Recovery- 55.9</td>
</tr>
<tr>
<td>Short term stock solution stability (8.35 hrs)</td>
<td>Felbamate Percent stability: 98.9% Zidovudine (IS) Percent stability: 97.9%</td>
</tr>
<tr>
<td>Long term stock solution stability (14.6 Days)</td>
<td>Felbamate Percent stability: 102.0 % Zidovudine (IS) Percent stability: 101.2%</td>
</tr>
<tr>
<td>In injector stability (24.00 hrs)</td>
<td>% Nominal: 95.1 - 106.9 % CV: 2.6 - 6.6 Percent stability: At LQC Level: 92.7 At HQC Level: 98.3</td>
</tr>
<tr>
<td>Dry extract stability (37.60 hrs)</td>
<td>% Nominal: 98.1 - 106.9 % CV: 2.6 - 5.3 Percent stability: At LQC Level: 95.6 At HQC Level: 94.8</td>
</tr>
<tr>
<td>Freeze – Thaw stability (4Cycles)</td>
<td>% Nominal: 100.6 - 105.4 % CV: 2.4 - 9.2 Percent stability: At LQC Level: 97.9 At HQC Level: 96.6</td>
</tr>
<tr>
<td>Bench top stability (9.00 hrs)</td>
<td>% Nominal: 102.4 - 105.4 % CV: 2.4 - 4.4 Percent stability: At LQC Level: 99.6 At HQC Level: 98.8</td>
</tr>
<tr>
<td>Long term stability in plasma (21.00 days at -20°C)</td>
<td>% Nominal: 101.9 - 108.7 % CV: 1.1 - 5.1 Percent stability: At LQC Level: 98.5 At HQC Level: 97.0</td>
</tr>
</tbody>
</table>
Based on the results obtained in this study, it is concluded that the present validated method can be successfully applied for the estimation of Felbamate in human plasma over the concentration range of 1.00 to 150.76 µg/mL. The method for determination of Felbamate in human plasma using HPLC with UV detection met the acceptance criteria with respect to selectivity, precision, accuracy, linearity, recovery and dilution integrity over a theoretical concentration range of 1.00 to 150.76 µg/mL for Felbamate.

Stability evaluations performed in EDTA human plasma, stock solutions and stock dilutions met the acceptance criteria, demonstrating insignificant degradation of Felbamate over the specified storage durations and conditions.
5) References


2. http://www.drugbank.ca/drugs,DB00949


