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Short communication

# Development of a high-throughput method for the determination of ethosuximide in human plasma by liquid chromatography mass spectrometry

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ABSTRACT

A simple, rapid, sensitive and specific ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method was developed and validated for the quantification of ethosuximide in human plasma is described. Analyte was chromatographed on a Hypersil Gold $C_{18}$ column (100 mm × 2.1 mm, i.d., 1.9 μm) with isocratic elution at a flow rate of 0.250 mL/min and pravastatin was used as the internal standard. The assay involves a simple solid-phase extraction procedure of 0.25 mL human plasma and the analysis was performed on a triple-quadrupole tandem mass spectrometer by MRM mode via electrospray ionization (ESI). The method was linear in the concentration range of 0.25–60.0 μg/mL. The lower limit of quantification (LLOQ) was 0.25 μg/mL. The within- and between-day precision and accuracy of the quality control samples were within 10.0%. The recovery was 95.1% and 94.4% for ethosuximide and pravastatin, respectively. The analysis time for each sample was 1.8 min. The method was highly reproducible and gave peaks with excellent chromatography properties.



## 1. Introduction

Ethosuximide [3-ethyl-3-methylpyrrolidine-2,5-dione] (Fig. 1), is an anticonvulsant succinimide. Anticonvulsant drugs are frequently used in the treatment of absence seizures unaccompanied by other types of seizures. Ethosuximide is suitable for use as a probe molecule and it can be given orally, its clearance can be estimated from a plasma or saliva sample [1]. The therapeutic monitoring of anticonvulsant drugs is rapidly becoming a routine aid in the clinical management of patients with epilepsy [2].

The use of LC coupled with electrospray tandem mass spectrometry has become the very popular technique in bioavailability studies due to the fast, sensitive, and reliable results generated by its use [3]. UPLC has been evaluated as a faster and efficient analytical tool compared to current HPLC [4]. Other techniques have been previously used for the quantitation of ethosuximide in human plasma. There are numerous published procedures that describe the quantitation of ethosuximide in plasma or serum. Different analytical methods for determination of ethosuximide as anticonvulsant drug have been reported, including high performance liquid chromatography (HPLC) [5–13], HPLC by derivatization [14,15] and gas chromatographic (GC) by chiral method [16,17]. To date only one LC/API-MS procedure has been reported for the quantitation of ethosuximide [18]. This method requires very low serum volume but has very high limit of quantification, longer chromatographic run time, SRM (selected reaction monitoring) mode, and protein precipitation extraction conditions. Herein we have reported a simple, and sensitive ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) method for the analysis of ethosuximide in human plasma by solid-phase extraction. The run time is only 1.8 min. The method is not only selective and sensitive but faster and simpler than any other reported method.

## 2. Experimental

### 2.1. Chemicals and reagents

Working standards of ethosuximide were between pravastatin and obtained from Synchron Research Services Pvt. Ltd. (Ahmedabad, India). High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Bangalore, India). HPLC grade of methanol, acetonitrile and analytical grade ammonium acetate were purchased from Merck Specialties Pvt. Ltd. (Mumbai, India). Drug-free (blank) human plasma was obtained from Supratech laboratory (Ahmedabad, India) and was stored at −20 °C prior to use. Oasis HLB® SPE cartridge used were obtained from Waters (Milford, Massachusetts).

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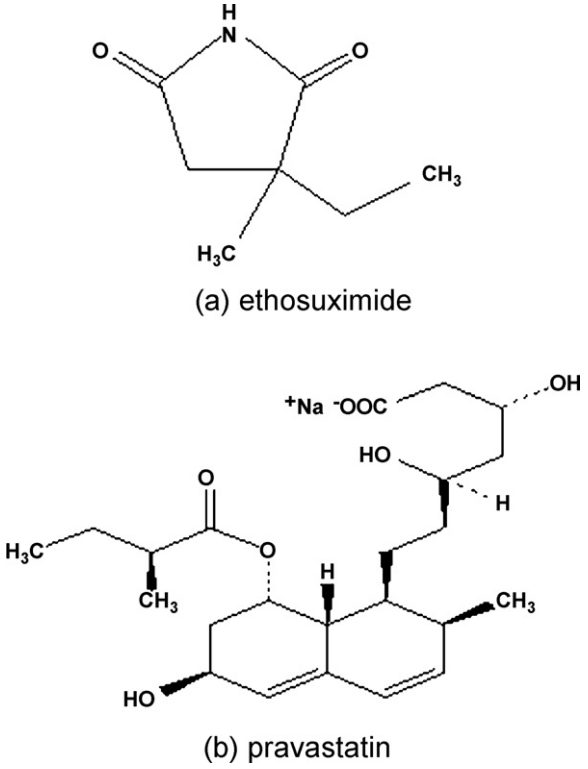


**Fig. 1.** Structure of (a) ethosuximide and (b) pravastatin.

### 2.2. Stock solutions and working standard

All stock solutions, calibration standards and quality control samples of ethosuximide (6.0 mg/mL) and pravastatin (IS) (3.0 mg/mL) were prepared with Milli-Q water. The stock solution of ethosuximide was serially diluted with Milli-Q water and these diluted solutions were used to prepare calibration curve and quality control samples. Nine-point standard calibration curve for ethosuximide (0.25–60.0 μg/mL) was prepared by spiking 0.5 mL blank plasma with 10 μL of appropriate amount of working standard solution. Quality control samples were prepared at four concentration levels of 0.26 μg/mL (LOQ), 0.5 μg/mL (low), 26.57 μg/mL for (medium) and 49.2 μg/mL (high) for ethosuximide. The stability studies for Quality control samples were spiked and stored at −20 °C.

### 2.3. Sample extraction

Two hundred and fifty microliters of human plasma was mixed with 25 μL of internal standard (680.0 μg/mL of pravastatin). To this 0.5 mL of Milli-Q water was added after vortex mixing for 30 s, the sample mixture was loaded in to an Oasis HLB extraction cartridge (30 mg/1 cc) that was pre-conditioned with 1.0 mL of methanol and equilibration with 1.0 mL of Milli-Q water. The extraction cartridge was washed with 1.0 mL of water. Analytes were eluted with 1.0 mL of mobile phase and 10 μL elute was injected in to the LC–MS/MS system.

### 2.4. Instrumentation and chromatographic conditions

Chromatography separation was performed on an Acquity UPLC™ system (waters corp, Milford, MA, USA) using Thermo Hypersil Gold $C_{18}$ column (100 mm × 2.1 mm, i.d., 1.9 μm) with cooling auto-sampler and column oven enabling temperature control of the analytical column. The column temperature maintained at 45 °C and chromatographic separation was achieved using mobile phase composition, acetonitrile–10 mM ammonium acetate (70:30, v/v) which was pumped at a flow rate of 0.250 mL/min. A partial loop with needle overfill was selected as an injection mode. The auto-sampler was maintained at 20 °C and the injection volume was 10 μL. Total run time for each sample analysis was 1.8 min. Mass spectrometric detection was performed using a waters (Waters Corp, Milford, MA, USA) Quattro Premier XE™ triple-quadrupole mass spectrometer with ESI in the negative ion mode. Ethosuximide and IS were quantitative determination by a multiple reaction monitoring (MRM) mode. The ion transitions monitored were $m/z$ 140 → 140 for ethosuximide and $m/z$ 423 → 101 for IS. The dwell time for each transition was set at 100 ms with an inter-channel pause time of 0.05 s to provide optimal sampling of each peak of interest. The optimal MS parameters obtained were as follows: capillary 3.2 kV, source temperature 120 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 700 and 20 L/h, respectively. Argon was used as the collision gas at a pressure of approximately $3.10 \times 10^{-3}$ mbar. The mass spectrometer, LC system, mass calibration, data acquisition, data representation and post-acquisition quantitative analyses were carried out using a MassLynx™ NT 4.1 software with a QuanLynx™ program (Waters Corp., Milford, MA, USA).

### 2.5. Method validation

Accuracy, precision, selectivity, sensitivity, linearity and stability were measured and used to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [19]. Each calibration curve contained a single set of calibration standards and six replicates of QC at four concentration levels. Selectivity was assessed, by comparing the chromatograms of six different batches of blank plasma obtained from six different sources with those of corresponding standard plasma samples spiked with ethosuximide and IS. Cross analyte effect (cross talk) was evaluated by spiking the LLOQ and ULOQ concentration with different lots (six normal plasma, one lipimic and one hemolyzed plasma) of plasma separately for analytes and I.S. Sensitivity was determined by analyzing six replicates of blank human plasma and plasma spiked with lowest level of the calibration curve. For the determining of within-day accuracy and precision a replicates ($n$ = 6) analysis of plasma samples were performed on the same day. The between-day accuracy and precision were assessed by analysis of three batches on different days. The precision was expressed as the relative standard deviation (RSD %) and the accuracy as the relative error (RE %). The recovery of ethosuximide and IS were determined by comparing the responses of the analytes extracted from replicates QC samples ($n$ = 6) with the response of analytes from post-extracted plasma sample at equivalent concentrations [20]. Recovery was determined at low, mid and high quality control concentrations, whereas the recovery of the IS was determined at a single concentration of 17 μg/mL. The matrix of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post-extracted plasma standard QC samples ($n$ = 6) with the response of analytes from neat samples at equivalent concentrations [20–21]. Matrix effect was determined at two levels (LQC and HQC) with six different source of plasma for ethosuximide and IS (at 17 μg/mL). Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Five replicates each at a concentration of double the uppermost calibration standard were diluted (2- and 10-fold) with blank plasma and analyzed. Process sample stability was evaluated with freshly spiked calibration curve and quality control

samples and those freshly spiked quality control samples compared with re-injecting the same sample which (stability samples) were stored at 20 °C for 48 h. Bench top stability was evaluated for 6 h at room temperature and compared with freshly spiked plasma samples. The freeze–thaw stability was determined by comparing the stability samples that had been frozen and thawed three times, with freshly spiked quality control samples. Long-term stability was evaluated by analyzing at low and high quality control samples those were stored at −20 °C for 60 days together with freshly spiked calibration and quality control standards. All stability evaluations were based on back-calculated concentrations. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% SD) and precision (i.e., 15% RSD) [19].

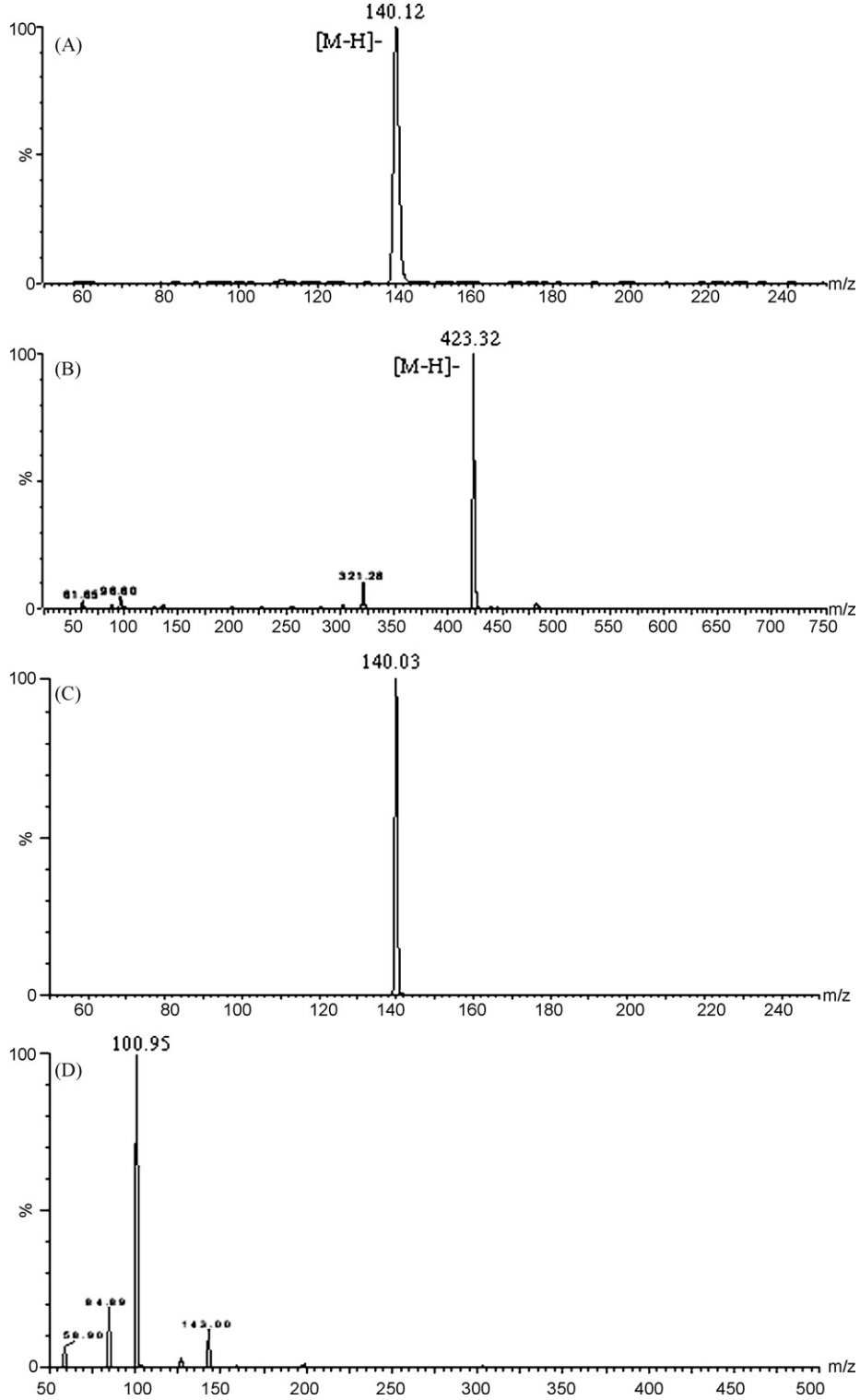


**Fig. 2.** Electrospray negative ion mass spectra of the precursor ion of (a) ethosuximide, (b) pravastatin and product ion of (c) ethosuximide, (d) pravastatin.

## 3. Result and discussion

### 3.1. Chromatography and sample preparation

A reversed-phase chromatographic method for the analysis of fat-soluble analyte was developed using ultra-high pressure liquid chromatography. Three different ACQUITY UPLC columns were studied for chromatographic separation: phenyl, octadecyl ($C_{18}$) and Shield RP18 BEH Shield. The phenyl column did not yield acceptable retention. The $C_{18}$ and Shielded RP18 columns allowed comparable separation of the analytes. The $C_{18}$ column was selected for the assay, because it furnished high sensitivity compared to the RP18 BEH Shield column. When combined with the UHPLC the $C_{18}$ reversed-phase technology allowed for an excellent separation at a flow rate of 0.250 mL/min and pressure of ~2000 psi (at 45 °C). Use of ammonium acetate in the mobile phase further enhanced the ion response with low background noise, resulting in higher selectivity. The composition of mobile phase, were optimized through several trials to achieve good resolution and increase the signal of analytes, as well as short run time. It was found that without adjusting the pH, the mixture of acetonitrile–10 mM ammonium acetate (70:30, v/v) could achieve this purpose and was finally adopted as the mobile phase. Under these conditions the chromatographic analysis of standard solution containing the present analytes showed a good separation of ethosuximide with retention time of 0.99 min and pravastatin with retention tine of 0.82 min, making the run time only 1.8 min. A simple solid-phase extraction was used in this assay. Different trials for SPE and liquid–liquid extraction (LLE) because this method was more convenient, rapid, sensitive, efficient, and reliable compared to LLE.

### 3.2. Mass spectrometry

The optimized MS conditions were performed using direct infusion of a methanolic solution of both positive and negative ionization modes for ethosuximide and pravastatin (IS) into the ESI source of the mass spectrometer and parameters such as capillary (ESI), extractor, and cone voltages were adjusted. However, with optimal MS tuning, a more consistent and higher response was achieved in negative ionization mode. Nebulizer and dessolvation gases were optimized to obtain better spray shape resulting in better ionization and droplet drying to form the deprotonated ionic ethosuximide and IS molecules. There was no major fragmentation observed for ethosuximide even after changing the collision energy but very high background noise observed without giving the sufficient product ion. The mass spectra for ethosuximide and IS revealed peaks at $m/z$ 140 and 423, respectively as deprotonated molecular ions $[M-H]^-$. Major fragment ion observed in IS product spectrum was at $m/z$ 101, respectively. The mass spectra of precursor and product ions of ethosuximide and pravastatin are presented in Fig. 2.

### 3.3. Specificity and selectivity

The method was examined by analyzing ($n = 6$) blank human plasma extracted again plasma spiked with the lowest standard. As shown in Figs. 3 and 4, no interfering peaks from endogenous compounds were observed at the retention times of analytes and IS. The total chromatographic run time was 1.8 min which provided a method with high sample throughput.

### 3.4. Matrix effect

The matrix effects in the LC–MS/MS method were evaluated by spiking blank plasma extracts with low and high QC samples. Average matrix factor values (matrix factor = response of post-spiked

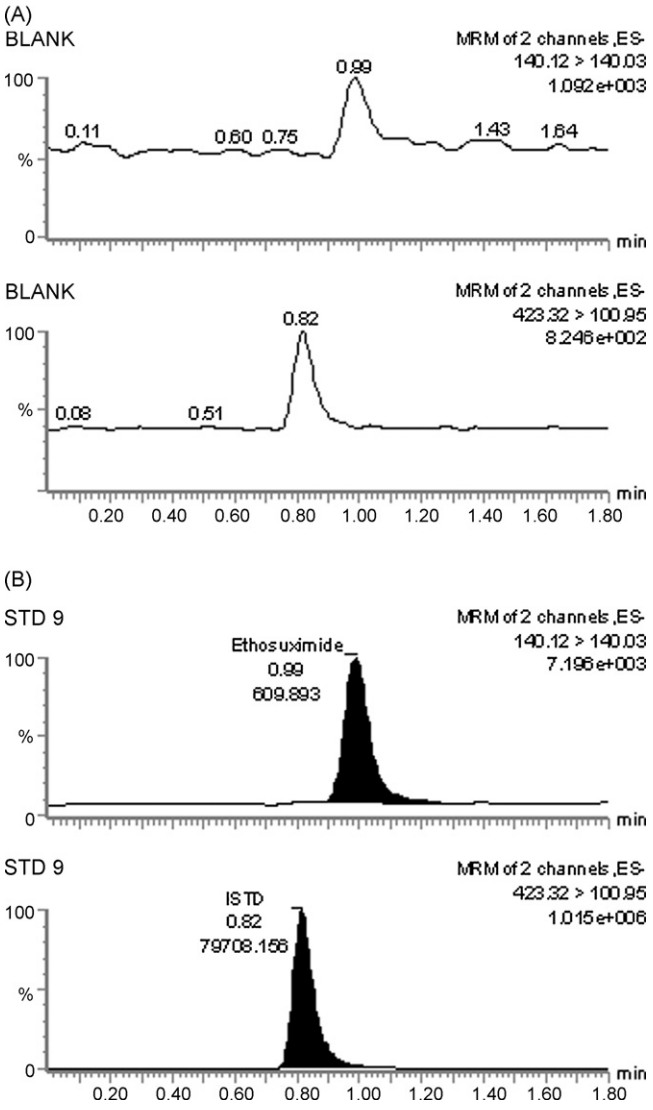


**Fig. 3.** Chromatogram of (a) blank human plasma, (b) plasma spiked with ethosuximide (0.25 μg/mL) and IS (680 μg/mL).

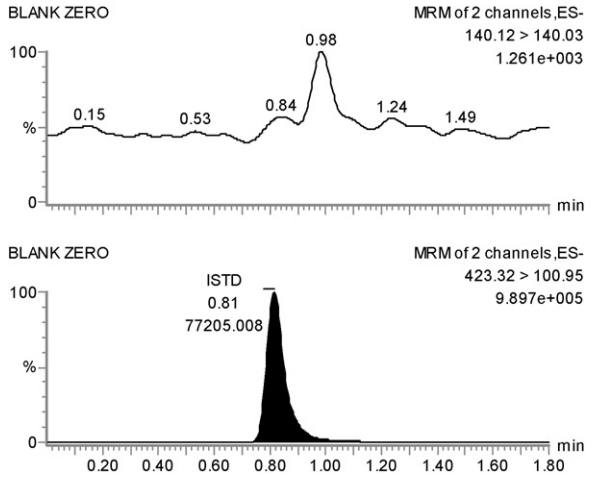


**Fig. 4.** Chromatogram of blank human plasma spiked with IS (680 μg/mL).

**Table 1**
Precision and accuracy data of back-calculated concentration of calibration samples for ethosuximide in human plasma.

| Analytes | Concentration added (μg/mL) | Concentration found (mean ± SD., μg/mL) | Precision (%) | RE (%) |
|---|---|---|---|---|
| Ethosuximide | 0.25 | 0.3 ± 0.01 | 3.6 | 2.2 |
| | 0.49 | 0.48 ± 0.04 | 8.1 | −3.6 |
| | 1.77 | 1.8 ± 0.06 | 3.6 | 2.9 |
| | 3.1 | 3.0 ± 0.03 | 0.8 | −1.7 |
| | 8.98 | 8.8 ± 0.25 | 2.9 | −2.4 |
| | 29.94 | 29.7 ± 0.36 | 1.2 | −0.7 |
| | 45.4 | 47.11 ± 1.10 | 2.3 | 3.8 |
| | 54.0 | 54.37 ± 1.18 | 2.1 | 0.7 |
| | 60.0 | 61.6 ± 1.03 | 1.7 | 2.7 |

**Table 2**
Within-day and between-day precision and accuracy for determining ethosuximide concentration in plasma samples.

| Analytes | Concentration added (μg/mL) | Within-day precision ($n$ = 6) | | | Between-day precision ($n$ = 18) | | |
|---|---|---|---|---|---|---|---|
| | | Concentration found (mean ± SD, μg/mL) | Precision (%) | RE (%) | Concentration found (mean ± SD, μg/mL) | Precision (%) | RE (%) |
| Ethosuximide | | | | | | | |
| LOQ QC | 0.26 | 0.3 ± 0.02 | 8.0 | −0.5 | 0.3 ± 0.03 | 9.9 | 2.8 |
| LQC | 0.5 | 0.6 ± 0.01 | 2.0 | 7.9 | 0.55 ± 0.04 | 8.0 | 3.1 |
| MQC | 26.57 | 26.92 ± 0.31 | 1.1 | 1.3 | 27.05 ± 1.49 | 5.5 | 1.8 |
| HQC | 49.2 | 51.35 ± 2.62 | 5.1 | 4.4 | 51.94 ± 2.23 | 4.3 | 5.6 |

concentrations/response of neat concentrations) obtained for ethosuximide were +0.97 (CV 5.2%, $n$ = 6) at LQC and +0.98 (CV 2.7%, $n$ = 6) for HQC, respectively, whereas on IS it was found to be +0.94 (CV 1.5%, $n$ = 6) at tested concentration of 17 μg/mL. There were no significant matrix effects observed for analyte or the IS.

### 3.5. Linearity

Nine-point calibration curve was found linear over the concentration range of 0.25–60.0 μg/mL was assessed by a weighted ($1/x^2$) least-squares regression analysis. The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of: $y = 0.019\ (\pm 0.001)x + 0.001\ (\pm 0.0001)$ where $y$ is the peak area ratio of the analyte to the IS and $x$ is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.99 or better; Table 1 summarizes the calibration curve results.

### 3.6. Precision and accuracy

The within-day and between-day precision and accuracy were within acceptable limits and are presented in Table 2.

### 3.7. Recovery

Five replicates samples at low, medium and high quality control were prepared for recovery determination. The mean recovery was 95.1 ± 4.4% and 94.4 ± 4.3 for ethosuximide and IS, respectively. The observed variability in % CV between these levels was 1.2% for ethosuximide and 2.7% for IS.

### 3.8. Dilution integrity

The upper concentration limits can be extended to 98.4 μg/mL by a 2- or 10-fold dilution with human plasma with a precision of 3.1% and an accuracy of 3.6%.

### 3.9. Stability

The stability of the analytes in human plasma under different temperature and timing conditions was evaluated and are enumerated in Table 3. QC samples were subjected to long-term storage conditions (−20 °C), and to freeze–thaw stability studies. All the stability studies were conducted at two concentration levels (0.5 and 49.2 μg/mL for low and high QC values) with five determinations for each. For process stability, the results indicated that the difference in the back-calculated concentration from time 0 to 48 h is 5.3%, which allowed us to conclude that processed samples

**Table 3**
Stability samples result for ethosuximide.

| Analytes | Stability | Spiked sample | Comparison sample | | Stability sample | | Mean % change |
|---|---|---|---|---|---|---|---|
| | | Concentration (μg/mL) | Concentration (mean ± SD, μg/mL) | Precision (%) | Concentration (mean ± SD, μg/mL) | Precision (%) | |
| Ethosuximide | Process[a] | 0.5 | 0.6 ± 0.02 | 3.4 | 0.5 ± 0.04 | 6.9 | −5.3 |
| | | 49.2 | 51.81 ± 1.56 | 3.0 | 53.44 ± 1.19 | 2.2 | 3.2 |
| | Bench top[b] | 0.5 | 0.59 ± 0.03 | 5.1 | 0.6 ± 0.04 | 7.5 | −0.3 |
| | | 49.2 | 51.6 ± 1.63 | 3.2 | 52.45 ± 2.29 | 4.4 | 1.7 |
| | Freeze and thaw[c] | 0.5 | 0.55 ± 0.01 | 2.6 | 0.57 ± 0.01 | 1.6 | 4.1 |
| | | 49.2 | 51.39 ± 1.11 | 2.2 | 52.15 ± 0.60 | 1.2 | 1.5 |
| | Long-term[d] | 0.5 | 0.5 ± 0.01 | 1.5 | 0.56 ± 0.01 | 1.6 | 5.9 |
| | | 49.2 | 52.51 ± 0.63 | 1.2 | 51.6 ± 1.63 | 3.2 | −1.8 |

[a] After 48 h in auto-sampler at 20 °C.
[b] After 6 h at room temperature.
[c] After three freeze and thaw cycles at −20 °C.
[d] At −20 °C for 60 days.

are stable at least for 48 h at 20 °C in the auto-sampler. For bench top stability, the results allowed us to conclude that both analytes are stable for at least 6 h at room temperature in plasma samples. Freeze and thaw stability results indicated that the repeated freeze and thawing (three cycles) did not affect the stability. Long-term stability evaluation brackets the time between the first sample collection and the last sample analysis. Long-term stability of the analytes in plasma at −20 °C was found to be stable for at least 60 days.

## 4. Conclusion

The method described here is a rapid, selective, sensitive and highly reproducible for the determination of ethosuximide in human plasma. The basic underlying advantage of this optimized method is that it utilizes only 0.25 mL of plasma, a very simple extraction procedure with no reconstitution step and short chromatographic run time of this assay are particularly suitable for routine assay. It was found to meet the requirements of US FDA guidelines for bioanalytical method validation. The simple solid-phase extraction method was used to extract the analyte and IS which provided excellent specificity and reproducibility. This validated method is suitable for the quantitative determination of ethosuximide in human plasma as well as pharmacokinetic and bioavailability studies of ethosuximide.

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