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Abstract: To quantify Cenobamate in human plasma, a precise and sensitive electron ionisation procedure in tandem mass spectrometry has been developed. The study was successfully confirmed by using Cenobamate -D4 as the internal norm and tert-butyl methyl ether in the testing of several positive ionic reactions. It has been used in liquid-liquid extraction to prepare samples as an extraction solvent. Cenobamate and Cenobamate – D4 (internal standard) were separated on an Eclipse C18 column (150 mm, 4.6 mm, 5 µm) by isocratic elution. This process was performed using a mobile phase of 0.1% formic acid in 20:80% v/v ratio of acetonitrile with a flow rate of 0.6 ml/min. The observed mass transitions for Cenobamate and D4 were m/z (amu) 268.23, 198.10 and 272.11 198.10 for each. The lower quantitative limit was 10 ng/ml based on 500 ng/ml plasma, with no chromatogram disturbance observed. A linear curve was observed with a correlation coefficient (r^2) of 0.999 from 10 to 500 ng/ml. The intraday and interday precision variations were 15% and the accuracy

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values were 15% of the relative error values (RE). Extraction recovery rates are in the tolerance ranges of less than 15%. The new LC-MS/MS method complies with all regulatory requirements and demonstrates satisfactory accuracy and accuracy and is sensitive enough to detect Cenobamate in humans.

Keywords: Cenobamate; human plasma; epilepsy; robustness; validation; development; LC-MS/MS.

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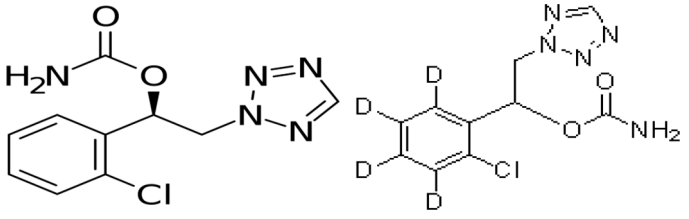
1 Introduction

Epilepsy is a chronic, non-transferable brain condition that affects people of all ages. It is primarily described as a random repetitive interruption in normal brain function called

epileptic seizures [1]. Chemically, Cenomabate [(1R)-1-(2-chlorophenyl)-2-(tetrazole-2-yl) ethyl], M.F $C_{10}H_{10}ClN_5O_2$, representing M.W 267.67 g/mol [2]. The chemical structures of Cenomabate and Cenomabate - D4 (internal standard) are shown in Figure 1. It is highly water soluble (1.7 mg/ml) with a log P value of 0.456 (and a pH of 7.4). For patients with partial convulsions, there was no adequate mechanism to measure Cenobamate efforts and therapeutic effects. In order to minimise repetitive neural firing by inhibiting stress-synchronised sodium currents, several studies have been published.

It is also a positive γ-aminobutyric acid (GABA) ion channel allosteric modulator [3]. Following an in-depth literature review, we observed that only one method of bioassay of CBM in rat plasma was available [4–6].

Figure 1 (A) Cenobamate and (B) Cenobamate-D4



We have therefore developed an accurate, accurate, and selectively optimised LC-MS/MS technique for quantifying CBM in biological fluids [7]. The aim of this study is to evaluate the Cenobamate by LC-MS/MS technique for quantifying CBM in biological fluids without violation of USFDA guidelines.

2 Materials and methods

2.1 Chemicals and reagents

CBM and CBM- D4 (IS) have been obtained from Ray Analytical Technologies, (Hyderabad, India) All chemicals (salts/solvents) used were LC-MS quality and purchased from Merck, Pvt. Ltd, India.

2.2 Instrumentation

The LC-MS/MS system consisted of an infinity LC Agilent 1220 spectrometer with automatic sampler that was made by Thermo Electron Corporation, USA fitted which use ESI ion source was used in the present study. To acquire and evaluate the details, Perkin Elmer and Simplicity 3Q were used. In the Eclipse C18 (150 mm, 4.6 mm, 5 µm) column, LC separation was conducted using isocratic elution with a 0.1% formic acid mobile phase: acetonitrile (20 : 80% v/v) at a flow rate of 0.6 ml/min. The temperature in the column stood at 80°C and the spectrometer was working at the positive ion mode. The voltage of the electric spray was set to 4500 V and 400°C was the capillary temperature. In multiple response monitoring (MRM) mode, ion detection was performed. Ionic transformation of m/z (amu) substance 268.23 198.10 to Cenobamate and 272.11 198.10 to internal standard.

2.3 Preparation of standard stock solution

A primary master solution was prepared by dissolving the calculated quantity of CBM and IS (internal standard) in the acetonitrile, sonified for 3 min, and prepared up to 10 ml with diluents [8].

2.4 Preparation of plasma sample

Samples for constructing the linearity curve were prepared as 10, 20, 40, 60, 80, 100, 200, 400, and 500 ng/ml of Cenobamate and each 50 ng/ml IS sample. Individual concentrations of quality control samples are 10 ng/ml (LLOQ), 30 ng/ml (LQC), 50 ng/ml (MQC), and 80 ng/ml (HQC) were prepared through injecting CBM to human plasma along with IS of 50 ng/ml. Then these samples are stored at 80°C.

2.5 Liquid-liquid extraction

200 µl of plasma and sample solvent were introduced into a glass tube after the plasma samples were dissolved at room temperature. 2 ml of diethyl ether, 10 µl of 0.1% formic acid were applied to each sample after shaking. The mixture was then whirled for 2min, then centrifuged for roughly 10 min at 3500 rpm [9]. In another clean glass tube, the organic phase was moved and vaporised under a slight nitrogen environment at 400°C.A 200 µl moving process, the residue was dissolved and moved into an automatic sampler bottle [10]. Finally, an aliquot was injected with LC-MS/MS.

2.6 Optimising chromatography conditions and mass spectrometry

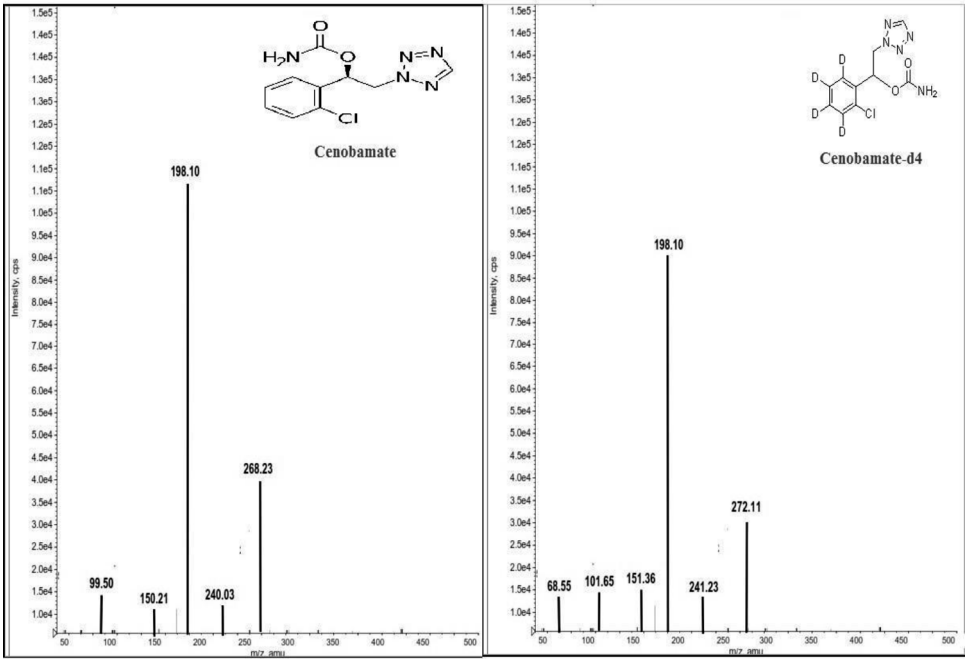
The evaluation of CBM and IS in human plasma was carried out by injecting 10 µl samples in the Eclipse C_{18} (150 mm, 4.6 mm, 5 µm), a mobile phase at a flow rate of 0.6 ml/min with a run time of 4.0 min. The column temperature was kept at the ambient temperature [11]. CBM was analysed in MRM positive ion mode with mass conversion of m/z (amu) 268.23, 198.10, and 272.11, 198.10. The mass spectra of parent and output ions are illustrated in Figure 2.

2.7 Optimising LC-MS/MS

The MS/MS optimisation was obtained through spiking CBM and IS solutions into the electrospray ionisation unit (ESI) of a steady mass flow rate spectrometer of 0.6 ml /min [12]. The ESI conditions were optimised with complete Quadrupole scans (Q1 scans) in a positive ion detection mode that produces the base peaks for CBM and SI at m/z (amu) 268.23→198.10 and 272.11→198.10, respectively. The mass terms are optimised **(**shown in table**)** to obtain the highest intensity of molecular ions of analyses the product ion of CBM showed the most abundant parent ion and production at m/z 268.23 and 198.10, respectively. However, the ion produced for CBM – D4 (internal standard) showed the parent ion and the ion produced most abundantly at m/z 272.11 and 198.10, respectively. The movement steps were optimised through several chromatographic experimental condition ways to obtain a tolerable retention time, preciousness, and similar peak for CBM and IS [13–15]. Finally, as a function of retention time, high sensitivity, maximum

symmetry, and reliability of the MS signal, 0.1% formic acid: acetonitrile (20 : 80% v/v) was chosen as the mobile step.

Figure 2 Mass spectrums of (A) Cenobamate and (B) Cenobamate-D4



(A) (B)

2.8 Validation of the analytical approach

Validation tests of plasma samples were performed in three consecutive days in three replications. Calibration curves were prepared and evaluated over three successive days to determine linearity. Calibration benchmarks were collected and analysed for nine Cenobamate concentrations at 10 ng/ml, 20 ng/ml, 40 ng/ml, 60 ng/ml, 80 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml, and 500 ng/ml values. The calibration curve was developed using the regression equation, Figure 3 to map the Cenobamate peak surface ratios relative to Cenobamate concentrations. The LLOQ calculation on use of five unique samples at different conditions shows the least concentration calibration curve with a high accuracy of ±20%. QC samples at four concentration levels (10, 30, 50, and 80 ng/ml) of CBM were tested and the on the five experimental trails for a period of three days to find the accuracy was shown in Table 1. Accuracy has been calculated by the relative standard deviation (percent RSD) measurement, Table 2. The expected matrix factor for the various individual samples counts the influence of the matrix (ME) and the constrict observations of the blank plasma sample are shown in Table 3. The linear data is shown as Figure 4.

Figure 3 MRM chromatograms of (A) Blank human plasma (B) Blank plasma spiked with Cenobamate-D4 (50 ng/ml) as internal standard (IS) (C) Plasma spiked with LLOQ standard (10 ng/ml) and Cenobamate-D4 (IS) 50 ng/ml) (D) Plasma spiked with U.L.O.Q (80 ng/ml) and Cenobamate-D4 (IS) 50 ng/ml) (see online version for colours)

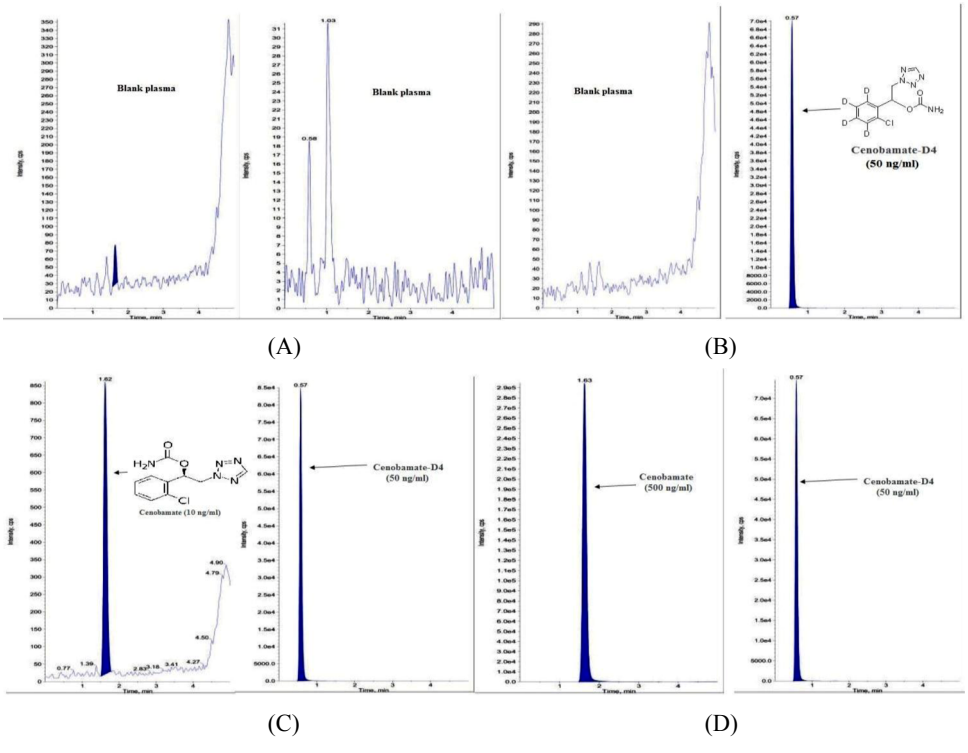


Table 1 Ruggedness data

Q.C levels	*Intra-day precision*		*Inter-day precision*	
	Mean ± SD	*Accuracy %*	*Mean ± SD*	*Accuracy %*
LLOQ (10 ng/ml)	9.78 ± 0.11	97.76	9.74 ± 0.21	97.37
LQC (10 ng/ml)	29.67 ± 0.33	98.89	28.51 ± 0.28	95.03
MQC (30 ng/ml)	247.06 ± 0.55	98.82	246.68 ± 0.4	98.67
HQC (10 ng/ml)	398.66 ± 0.83	99.67	395.51 ± 2.85	98.88

QC – Quality control; Mean of six determinations (n = 6).

Table 2 Robustness data

Parameter	*Flow rate (+5%) ml*	*Flow rate (–5%) ml*	*Column temp high (+2°C)*	*Column temp low (–2°C)*
Mean peak area**&SD	251.1 ± 0.76	250.86 ± 1.06	251.154 ± 0.54	251.95 ± 1.20
% RSD	0.30	0.42	0.21	0.48
Mean % RSD	0.36		0.35	

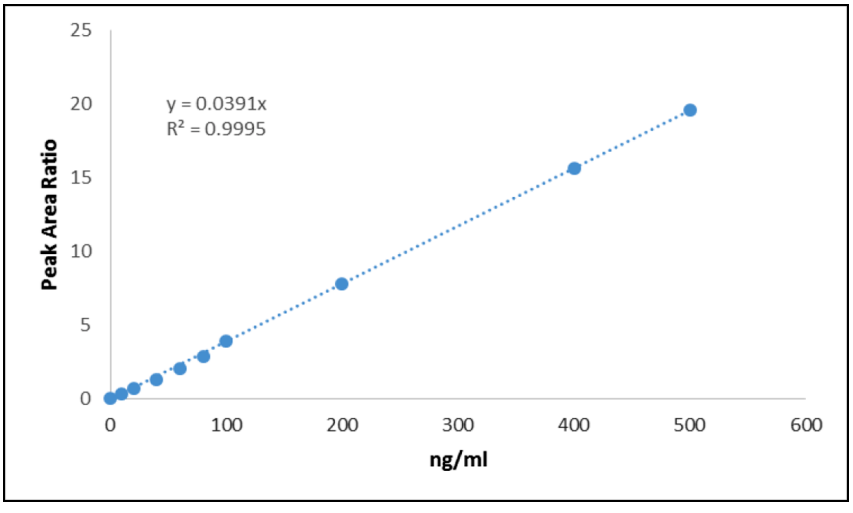
**Mean of six determinations.

Table 3 Accuracy studies

Parameter	*Cenobamate*	*Cenobamate-D4*
LQC		
Mean peak area*	16,360 ± 16.84	330,957 ± 190.82
% RSD	0.10	0.06
% Recovery	89.20	93.59
MQC		
Mean peak area*	924,621 ± 54.25	330,753 ± 190.82
% RSD	0.01	0.06
% Recovery	90.30	93.00
HQC		
Mean peak area*	18,78,320 ± 263.79	328,780 ± 4209.88
% RSD	0.01	1.28
% Recovery	94.94	91.66
Mean % recovery	91.48	92.75
Mean % CV	0.05	0.71

*Average of six determinations.

Figure 4 Linearity data (see online version for colours)



Repeated analysis ($n = 3$) of the three-level QC samples gave LQC (89.20), MQC (90.30) and HQC (91.48). The matrix factor's overall precision was expressed as a variance coefficient (cv percent) and 15% CV. The short-term viability of the Cenobamate was measured by calculating the QC samples stored for 24 h at ambient condition. The sustainability was measured by assessing the quality control samples deposited for one

month at a low temperature (80°C). The stability of post- preparation was calculated by determining the quality control samples stored for 24 h under automated sampler (4°C) conditions [13–15]. The freeze and thaw stability was examined under the freeze condition at 80°C and thaw at the ambient temperature for a period of five days. Table 4 displays the sustainability of the stock solutions for Cenobamate and IS which was examined through the study at 4°C. Table 5 illustrates the permanence of the Cenobamate in plasma under different storage conditions.

Table 4 Stability data of standard solutions

Parameter	*Cenobamate*		*Cenobamate-D4*	
	**Comparison samples*	**ST Stability samples*	**Comparison samples*	**ST Stability samples*
Mean peak area*	910,419.67 ± 105,28.43	854,746.3 ± 37,847.16	397,709.67 ± 3385.60	327,381.67 ± 12 069.39
CV (%)	1.16	4.43	0.85	3.69
Mean % accuracy	98.51%		95.54%	

*Mean of six determinations.

2.9 Detection of LOD and LOQ

By comparing the LOD and LOQ concentrations with known analysis of blank plasma samples were determined using a signal-to-noise ratio (S/N).

CBM and CBM-D4 were analysed at three spiking levels (LQC, MQC, and HQC) into blank plasma samples. The accuracy studies were done by determination of peak area compared with the standard samples from CBM and IS. The RSD of every level is <15%.

2.10 Stability studies for standard solutions

Previously prepared stock solutions of CBM and IS preparations were subjected to different conditions as described earlier to study the stability of the analytes.

2.11 Sample stability

The stability of CBM in plasma was analysed at ambient conditions for a period of 24 h using LQC and HQC samples and the above solutions were stored for 30 days at a temperature of –80°C to verify the sustainability of CBM in plasma under various testing conditions. The result was obtained as recovery % rate and standard deviation, under testing the stability sample with freshly spiked samples and the observations are compared. The amount found is within the acceptance criteria of 85–115% and 80 ng/ml, RSD is ±15%.

Table 5 Sustainability of Cenobamate inside plasma under various testing conditions

Quality control sample	*Concentration of Cenobamate (ng/ml)*	
	*LQC**	*HQC**
Freeze and Thaw stability (–80°C)		
% CV	13.68	2.07
% Accuracy	91.41	98.17
Room temperature stability (24 h)		
% CV	1.62	2.15
% Accuracy	95.18	94.95
Auto sampler stability (24 h)		
% CV	2.47	1.83
% Accuracy	94.77	97.00
Re-injection stability (40 h)		
% CV	8.09	1.61
% Accuracy	94.32	98.22
Long term stability (1 month; –80°C)		
% CV	1.79	2.80
% Accuracy	94.24	97.75

*Average of six determinations.

3 Results and discussion

3.1 Process development and optimisation

The objective is to develop and examine a sensible method for extracting and quantifying CBM in human plasma. In developing the method, a different chromatographic parameters were measured and examined to predict the optimise sample extraction. The MRM positive ionic mode mass spectroscopy parameters such as collision energy of the collision gases have been optimised so that the basic peaks for CBM and IS have to be found at m/z 268.23→198.10 and 272.11→198.10, respectively.

Chromatographic conditions, including mobile phase composition, were optimised by changing the ratio of formic acid to acetonitrile by 0.1% by various test cases to acquire suitable preservation time, adoptability and symmetric peak geometry and signal stability for CBM and IS.

3.2 Specimen preparation and IS selection

The sustainability of the post-preparation sample quality control was deposited under automatic sampler at a working temperature of 4°C about 24 h. This process will measure the freeze and thaw sustaniability by measuring the QC samples that are been deep freezed to 80°C and the thaw which was tested at the ambient temperature over following

days. The sustainability of the stock solutions for Cenobamate and IS was calculated by an analysis carried out at 4°C, Table 4, of their work solutions.

3.3 Method validation

The method's accuracy was tested by evaluating individual white plasma samples from six different sites. During the retention of an analyte or IS, all tests showed no interference with endogenous compounds. For CBM and IS, the retention period was around 1.65 ± 0.05 min and 0.58 ± 0.05 min. Typical chromatograms from plasma to white, a plasma doped with standard CBM and IS sample were noted. This method demonstrated an excellent linear response in the considered concentration range of 10–500 ng/ml with an r^2 value of 0.999.

The LLOQ for CBM was proved to be 1.48%. The accuracy and precision were performed to the LSD considered factors such as: LLOQ, LQC, MQC, and HQC, the acceptable criteria for inter and intraday precision in % RSD (Relative Standard Deviation) should be less than or equal to 15% for LQC, MQC, HQC and 20% for LLOQ samples.

Mean extraction recoveries for CBM and IS were measured at three separate concentrations of LQC, MQC, HQC. The peak regions of the CBM spiked in plasma are compared with direct injection of dissolved standards which was treated under blank plasma supernatant. At each QC stage (30, 50, and 80 ng/ml), mean extraction recoveries were found to be $60.3 \pm 3.3\%$ and $70.3 \pm 6.1\%$, respectively, and endogenous co-elution compounds were the extraction recovery of IS. The quality of ionisation can be impaired. The concentrations collected from the mobile phase norms was compared peak regions of CBM samples and it was found to be compactable. Ratios of 30 ng/ml, 50 ng/ml, and 80 ng/ml concentrations of LQC, MQC, and HQC. These results showed that in our analysis, the matrix effect did not have a major effect on the efficiency of the assay. The stability study results indicate that CBM was not subjected to a significant degradation in any of the conditions implemented to study the stability of the analyte. The % RSD of all the stability parameters were found to be within the limits.

4 Conclusion

In this analysis, using a basic liquid-liquid extraction technique, an LC-MS/MS approach was developed and measured the effect of CBM in human plasma. LC-MS/MS (liquid chromatography with tandem mass spectrometry) process provide better sensitivity than previously published methods. The calculation is performed by understanding the variability, specificity, repeatability, and matrix effects of the test case samples. This invented approach was satisfactory within its reasonable limits as per the USFDA guidelines.

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Conflict of interest

Authors assuring that, there is no conflict of interest.

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Abbreviations

USFDA	United States Food and Drug Administration
LC-MS	Liquid chromatography mass spectroscopy
LLE	Liquid–liquid extraction
ESI	Electrospray ionisation
CBM	Cenobamate
IS	Internal standard
RE	Relative error
RT	Retention time
CAN	Acetonitrile
LLOQ	Lower limit of quantification
LQC	Lower quality control
MQC	Middle quality control
HQC	High quality control
LOD	Limit of detection
LOQ	Limit of quantification