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## Quantitation Of Acamprosate In Human Plasma By LCMS And Their Application For Pharmacokinetics Parameters In Bioequivalence Study

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### ABSTRACT

The purpose of this research and development was to develop the elevated susceptible, speedy, stable and reproducible extraction method with precise results. At the same time, this method would be efficient in analyzing the large numbers of plasma samples obtained for bioequivalence studies. The chromatography was performed by using column ZIC<sup>®</sup>-HILIC, 4.6×150 mm, 5μ. Nimodipine was used as internal standard. The solid phase extraction was used to extract Acamprosate and Nimodipine (Internal standard) from plasma samples. The calibration curve was relied on the concentration of 10.01 ng/mL to 709.36 ng/mL for Acamprosate in human plasma

**Keywords:** Bioequivalence study, LCMS and solid phase extraction

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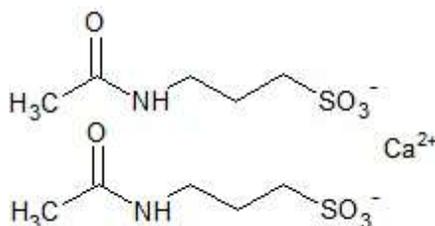
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## INTRODUCTION

Acamprosate Ca is to stabilize the chemical balance in the brain. It will be disrupted by alcoholism, possibly by blocking glutaminergic *N*-methyl-D-aspartate receptors, while gamma-aminobutyric acid (GABA) type A receptors are activated [1]. It is an antidipsotropic agent that was approved by the US Food and Drug Administration (FDA) in 2004 for use in alcoholic individuals to decrease alcohol hankering after alcohol detoxification [2]. A monograph on Acamprosate Calcium is being introduced by Lipha involve a potentiometric titration for the assay of the drug and HPLC method for the determination of Homotaurine, which is both precursors of the synthesis and a potential degradation product of Acamprosate [3].

Acamprosate has been commercially available there since from 1989, in 333 mg tablet strength [4]. A literature survey showed that capillary zone electrophoresis methods have been developed for the dissolution and related compounds of Acamprosate tablets [5-6]. Several authors are reported bioanalytical methods for the analysis of Acamprosate as a single drug in human plasma, dog plasma, and urine. [7-12].

Acamprosate calcium is a white, odorless or nearly odorless powder. It is freely soluble in water and practically insoluble in absolute ethanol and dichloromethane. Its chemical formula is  $C_{10}H_{20}N_2O_8S_2Ca$  and the molecular weight is 400.48.



**Figure 1: Structure of Acamprosate calcium**

A detailed literature survey reveals capillary zone electrophoresis methods [6-7], bioanalytical methods for the analysis of Acamprosate calcium using LCMS [8-16], LC-fluorometric and Electrochemical detection [8] in human plasma, dog plasma, and urine. Recently, two RP-HPLC methods have been reported for the quantitative estimation of Acamprosate calcium in tablets [17-18].

We report a totally new, precise, linear and accurate isocratic LCMS method for the quantitative estimation of Acamprosate calcium in ACAMPROL.

Compliance with good laboratory practices (GLP) for conducting sample analysis of nonclinical (also known as preclinical) laboratory studies and clinical studies are intended to ensure the quality and integrity of the safety data filed in support of investigational new drug applications (NDAs),

new drug applications (INDs), abbreviated new drug applications (ANDAs), supplements in developing bioanalytical method validation information used in human clinical pharmacology, pharmacokinetics and pharmacodynamic study), [1-7] in varieties of matrices like human plasma [5-8], and dog urine [9], dog plasma [10]. Among all reported methods LC-MS method gives the best results. Ghosh C. *et. al.* explained more about the matrix effect of Acamprosate in biological matrices and they developed the method by using precipitation extraction method and reported for quantitation with linearity range 7.04 to 702.20 ng/mL using LCMS in Human Plasma. Hammerberg *et. al.* reported method in human as well as in CSF (Cerebrospinal Fluid) by using LCMS/MS and quantifying the linearity range between 9 to 33 ng/mL in CSF. Rhee *et. al.* reported method in dog plasma by precipitation method with LCMSMS.

The purpose of this development is to give highly sensitive, selective, reproducible extraction method for the long term with selective linearity range. At the same time, this method would be efficient in analyzing a large number of plasma samples obtaining from the pharmacokinetic bioavailability and bioequivalence study.

## MATERIALS AND METHOD

Acamprosate and Nimodipine obtained from Vivan life science Mumbai India. Methanol and acetonitrile were purchased from the local distributor of Finar with LC Grade. Ammonium formate and formic acid purchased from the S D. Fine with AR Grade. Drug-free human plasma was procured from Amclin Life Sciences, Pune.

### Instrumentation

Mass Spectrometry API 4000 triple quadrupole instrument from (AB Sciex) frontend was used as Ultra Performance Liquid Chromatography from Shimadzu. Data processing was done by using Analyst software 1.6.3

### Mass Parameters

The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Sample introduction and ionization were in the negative ion mode. Sources dependent parameters were as follows. Ion Spray Voltage was 4500 and temperature was 400. The compound dependent parameters such as declustering potential (DP), Focusing potential (FP), Entrance potential (EP), Collision Energy (CE) and cell exit potential (CXP) were optimized during tuning as 60, 400, 12, 30 and 15 eV for Acamprosate and 40, 400, 8, 30 and 5 eV for Nimodipine respectively. The collision activated dissociation (CAD) gas was set at 3 psi by using nitrogen gas. Dwell time was set

at 200 msec for Acamprosate and Nimodipine. The mass transition was selected as 179.9 – 80.0 for Acamprosate and 417.0-122.0 for Nimodipine.

### **Preparation of Stock Solutions And Standards**

Acamprosate stock solution prepared by weighing 27.2 mg of Acamprosate calcium (equivalent to 25 mg Acetyl homotaurinate) of working standard and transferred into 25 mL volumetric flask add 10 mL of water and sonicate to dissolve it and make up the volume with water. (886692.92 ng/mL) Calibration standard were prepared at concentration levels of 10, 20,30,55,91,153,255 and 425 ng/mL and quality control samples were prepared at concentration level of 30,108, 217 and 396 ng/mL.

Nimodipine stock solution prepared by weighing 25 mg of Nimodipine and transferred into 25 mL volumetric flask add 10 mL of methanol and sonicate to dissolve it and make up the volume with methanol. (1000µg/mL) 0.025mL of Nimodipine stock solution transferred to 50 mL volumetric flask and volume make up to 50mL with diluents (Water : Methanol::50:50)

### **Sample Preparation**

A hybrid method is used for separation of drug from plasma. Acetonitrile used for precipitation and solid phase extraction is used for separation of drug from plasma. Take 300 µL of plasma into the RIA vial add 50 mL of Internal standard then vortex approximately followed by 0.5 mL of 0.1% formic acid in acetonitrile added and vortex for 2 minutes. These samples were loaded to SPE cartridges Orochem ICC phospholipids cartridges which were preconditioning with 1mL methanol followed by 1 mL water. The supernatant liquid of samples was loaded and eluted liquid directly transferred to the autosampler vial and 5µL samples injected from the autosampler vial in optimized chromatographic condition.

### **METHOD VALIDATION:**

#### **Selectivity**

Six different screen lots of human plasma along with haemolyzed and lipemic plasma collected from the different donors for selectivity. This screened plasma used for validation experiments to test for interference at the retention time of analyte and IS.

#### **Matrix Effect**

The matrix effect due to the plasma used to evaluate the ion suppression/enhancement in a signal when comparing the response of QC samples after pretreatment. This experiment was performed by spiking the LQC and HQC in six different replicates of blank plasma samples with satisfactory precision. (< 15%)

#### **Sensitivity**

Sensitivity was evaluated at LLOQ level by injecting six extracted samples in K3EDTA based human plasma.

### **Carryover Test**

This test was performed by injecting the AQ LLOQ and AQ STD H along with extracted LLOQ and STD H samples.

### **Precision and Accuracy**

It was determined by replicate analysis of quality control samples (n=6) at LLOQ (Lower limit of Quantitation), LQC (Lower Quality Control), MQC (Middle-Quality Control) and HQC (Higher Quality Control) samples. Precision and accuracy should be within 15% except for LLOQ, for LLOQ it should be within 20%.

### **Recovery**

Recovery experiment was carried out on three levels, LQC, MQC, and HQC and for the internal standard, it was proved at a single concentration at respective standards.

### **Injection Reproducibility**

This exercise was done to assess any change occurring due to the reacquisition of the same samples in the vials which were kept in the Autosampler. Reinjection Reproducibility was evaluated by preparing and extracting a set of Calibration Curve Standards and six replicates of LQC and HQC samples

### **Dilution Integrity**

During the subject sample analysis, some unknown sample concentration may fall above the ULOQ and below the MQC level. To estimate the actual concentration of those unknown samples, dilution integrity test was performed at 1.5 times of ULOQ concentration and performed at six replicates from each level (1/2 and 1/10 of ULOQ) and calculate by applying dilution factor 2 and 10 with freshly prepared standards.

### **Haemolised and Lipemic Effect**

Haemolyzed matrix has a lot of natural variabilities. Haemolysis and lipemic effect can affect the response of Analyte during the method validation and subsequently in subject analysis. The quantification of Analyte from plasma can be grossly affected by a significant haemolysis and lipemic effect. This analysis performed by using the LQC and HQC Level.

### **Long Batch Performance**

The Long Batch Performance experiment was performed to enable evaluation of any trends over time within one run. This exercise was done to assess the performance of the instrument in the analytical run during the proposed analysis of subject samples of the bio-study.

### **Ruggedness**

The ruggedness test was evaluated with different Analyst, different Column and different Equipment. This test is performed by using a precision and accuracy batch.

### **Stability**

Stability of drug demonstrated in stock solution and in plasma samples stability of internal standard was proved in the stock solution. Stability of drug and internal standard was evaluated by comparing the area values of the freshly prepared and stable solution. Stability of drug plasma was performed by using spiked LQC and HQC samples. Short term stability, long term stability, bench top stability, autosampler stability, and freeze-thaw stability were proved on plasma samples for Analyte.

## **APPLICATION OF METHOD**

### **Analysis of plasma samples**

The above-mentioned bioanalytical method described to analyses the homotaurinate (Acamprosate) from the human plasma. This method applied to study the oral dose of healthy volunteers. These volunteers were contracted at Raptim research ltd. Mahape, New Mumbai India. Test product Acamprosate tablets 333mg given to the 14 healthy male volunteers with 240 mL of water. The reference product is Campral® (Acamprosate calcium) Delayed-Release Tablets 333 mg manufactured by Forest Pharmaceuticals Inc., Subsidiary of Forest Laboratories, Inc., St. Louis, Missouri 63045. The study protocol was approved by the ethical committee and also by DCGI. Blood samples were collected at the following time points. Pre-dose sample (collected within 1 hour prior to drug administration), 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00,6.50, 7.00, 7.50, 8.00, 8.50, 9.00, 9.50, 10.00, 10.50, 11.00, 12.00, 16.00,20.00, 24.00, 48.00, 72.00 and 96.00 hours (post-dose) within 2 minutes of scheduled sampling time (except for ambulatory sample which was collected up to 24.00 hours from the scheduled time). Blood samples were collected in pre-labeled K3EDTA vacutainers. Total 29-time points for Test and Reference were collected. Plasma was separated from this blood samples by rotating these samples at 2-8°C and 5000 rpm. The plasma was separated and transferred to the prelabeled RIA vial. These samples were stored at -20 °C for short duration and for long duration stored in -70 °C storage deep freezer.

### **Pharmacokinetics and statistical analysis**

Winnonlin and SAS statistical applications were used to study the Pharmacokinetics parameter from human plasma samples. The non compartmental statistical model was used in the winnonlin software. Blood collection was done up to the elimination of half-life of the drug from the human and it was calculated as area under the concentration-time curve. Primary parameters  $C_{max}$ ,  $AUC_{0-t}$ ,  $AUC_{0-\infty}$  and secondary parameters  $t_{1/2}$ ,  $K_{el}$ ,  $T_{max}$  were calculated from the statistical application.

The terminal elimination rate constant ( $K_{el}$ ) was estimated from the slope of the terminal exponential phase of the plasma of Acamprosate concentration-time curve. The primary and secondary parameters were calculated by means of ANOVA and calculating the 90 % confidence interval of the test to reference ratios (logarithmically transformed data). The bioequivalence was considered when the ratio of log-transformed data was within the 80-125 % for primary parameters  $C_{max}$ ,  $AUC_{0-t}$ ,  $AUC_{0-\infty}$ .

## RESULTS AND DISCUSSION

### Method Development

During method development mass parameter optimization, chromatography optimization, suitable extraction method optimized before initiate the method validation

### Mass Parameter Optimization

During mass parameter optimization type of ionization is a major factor to get the respective parent and product ions. In Acamprosate analysis Electron spray ionization (ESI) was selected for ionization. Compound dependent (DP, FP, EP, CE & CXP) and source dependent (CUR, CAD, heater gas, nebulizer gas) temperature, voltage condition parameters were tuned to get the better signal and response of the analyte and internal standard. Acamprosate response was more in negative ion comparative to positive ionization mode. Internal standard Nimodipine corresponds to the masses 417.00 and product ions 122.00, all other parameters to get the more intensity are tuned.

### Chromatographic Parameters Optimization

During the optimization of chromatography, selection of suitable mobile phase and the column is the initial aspects. The mobile phase containing ammonium acetate, acetic acid, Methanol and acetonitrile in varying combinations were tried. The low response, poor chromatography, low sensitivity was observed. Finally, the mobile phase was selected to 10 mM ammonium format: Acetonitrile (30:70) v/v was selected for good sensitivity response of analyte and internal standard with good chromatography.

Different columns like Agilent 15cm, 4.6mm 5 $\mu$ , Inertsil ODS with the same specification also use the column with 5cm, 4.6mm 5 $\mu$  but the chromatography was not found acceptable. Good chromatography was observed in ZIC<sup>®</sup>-HILIC, 4.6 $\times$ 150 mm, 5 $\mu$ . This column was selected for further analysis; it gives a satisfactory response for both Acamprosate sodium and Nimodipine at a flow rate of 1.0 mL/min with 80% splitter with a run time of 3.50 mins. Analyte Acamprosate was eluted at retention time 2.21min.

For LCMS analysis, utilization of stable isotopes or deteriorated or same structural activity compound use as an internal standard to correct the matrix effect is needed. In this method, Nimodipine was used as an internal standard. The column temperature was adjusted to 25°C and injection volume 5  $\mu$ L was used for better chromatography.

### Extraction Method Optimization

During the optimization of the extraction method following procedures were followed by protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction. We found less response in the LLE method, we tried for precipitation and SPE method for further analysis. We have a problem to inject the precipitated sample through the LCMS, so this precipitated sample to pass through the SPE cartridge to get the clean sample for analysis. This extracted sample does not carry any foreign material to create the problems in analysis. High recovery and selectivity were observed in this hybrid method. This optimized detection parameters, chromatographic conditions, and extraction procedure resulted in reduced analysis time with an accurate and precise method for detection of Acamprosate in human plasma.

### METHOD VALIDATION

Method validation of Acamprosate in human plasma was performed as per the USFDA guidelines. This method is validated for selectivity, sensitivity, matrix effect, linearity, precision, recovery, dilution integrity reinjection reproducibility, and stability.

### Selectivity and Sensitivity

There is no interference observed for Acamprosate and Nimodipine at their retention time in blank plasma (fig2 and fig 3). The selectivity was accepted for all six batches injected along with haemolyzed plasma. The LLOQ of Acamprosate was 10 ng/mL The precision and accuracy of the LLOQ plasma samples was found 5.17 and 99.70 respectively.

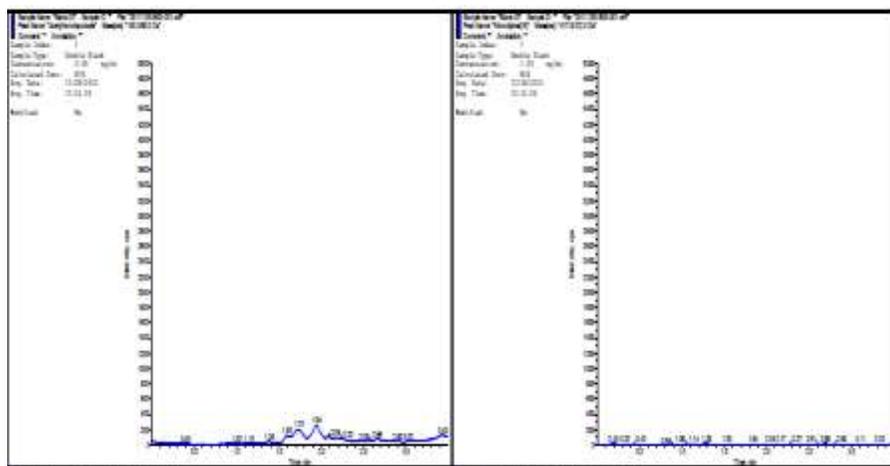
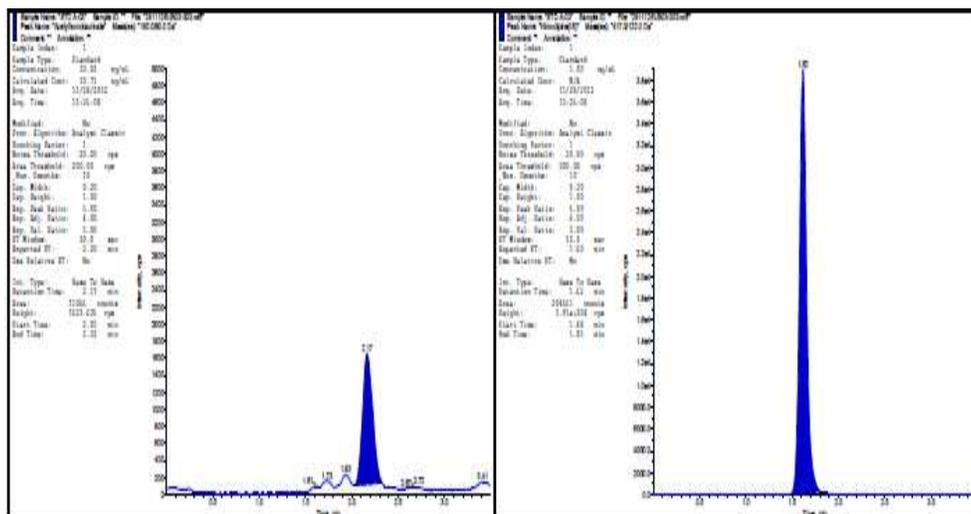


Figure 2 Blank for Analyte and Internal Standard



**Figure 3 Chromatogram for LOQ and Internal Standard peak**

### Matrix Effect

The % RSD for ion suppression and enhancement in the signal was found to be 6.10 & 2.08 at LQC and HQC level for Acamprosate respectively indicating that the matrix effect on the ionization of analyte is within the acceptable range.

### Linearity

The calibration curve was plotted as the peak area ratio versus Acamprosate concentration, it was found to be linear over the concentration range of 10.01 ng/mL to 709.36 ng/mL. The precision was found 0.69% to 13.87 % and the accuracy was found 95.80-100.21%. The correlation coefficient was greater than 0.9975 for all calibration curves. (Table-1)

**Table-1 Calibration Curve Details**

Concentration (ng/mL)	Mean±SD	% CV	Accuracy
10.01	9.77±1.37	13.97	97.60
20.02	19.56±1.13	5.80	97.70
40.04	40.06±1.70	4.25	100.05
80.08	76.72±2.97	3.87	95.80
143.01	139.78±3.13	2.24	97.74
255.37	271.64±8.40	3.09	106.37
425.61	415.13±11.48	2.77	97.54
709.36	710.83±4.94	0.69	100.21

### Precision and Accuracy

Precision and accuracy for this method was calculated by on the basis of inter-day and intra-day variation at four concentrations (LQC, LMQC, MQC, and HQC concentrations are 30.01, 63.84, 319.21 and 638.42 ng/mL respectively) in six replicate injections. Inter-day precision was found less than 5.88% and accuracy was found 98.48-100.11%. Intraday precision was found less than 6.81%

and accuracy was found 96.06-98.33%. All these results mentioned in the Table-2 and 3 this showed that this method is precise, reliable and adequate between the calibration curve range.

**Table-2 Inter-day precision and accuracy**

Concentration (ng/mL)	Mean±SD	% CV	Accuracy
30.01	29.59±1.74	5.88	98.60
63.84	62.87±3.23	5.14	98.48
319.21	315.07±12.04	3.82	98.70
638.42	639.11±37.21	5.82	100.11

**Table-3 Intra-day precision and accuracy**

Concentration (ng/mL)	Mean±SD	% CV	Accuracy
30.01	29.51±2.01	6.81	98.33
63.84	61.92±3.89	6.29	96.99
319.21	308.22±13.13	4.26	96.56
638.42	613.26±29.62	4.83	96.06

### Recovery

Recovery was calculated by comparing the area observed in the plasma samples against the area observed in the solvent samples. Recovery was determined on three different levels (LQC, MQC, and HQC). Recovery was found 77.69, 82.89 and 79.11 % respectively. The average recovery for the analyte and internal standard was found to be 79.90 and 68.67 % respectively.

### Dilution Integrity

The dilution integrity was calculated for the concentration of ½ and 1/10 diluted samples and found within the acceptance criteria. The % RSD was found 2.09-2.28% and % nominal was found 99.47-100.71% respectively.

### Stability

Quantitation of plasma samples feasible after 4 plasma freeze-thaw cycles (-20°C- room temperature) analyte was found to be stable at four cycles. No significant degradation was observed in the short term (6 hrs) and long term (7 days) in Acamprosate and Internal standard (Nimodipine) standard solutions. There was no significant degradation was observed in the autosampler stability, bench top stability and long term stability (Refer Table-4). These results prove the stability of Acamprosate in human plasma.

**Table-4 Stability**

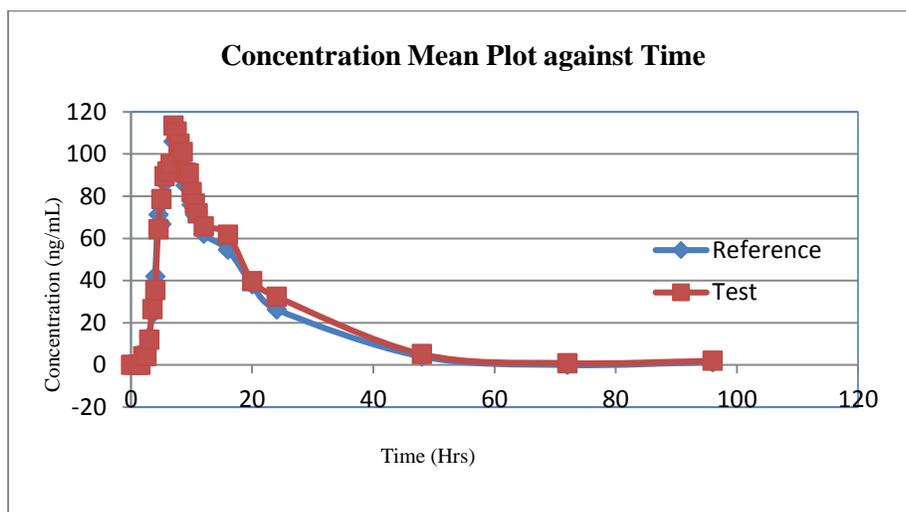
Stability Experiment	Concentration (ng/mL)	Mean±SD	% CV	Accuracy
Freeze-Thaw Stability (Cycle-4)	30.01	29.49±0.50	1.69	98.27
	638.42	593.90±22.60	3.80	93.03
Autosampler Stability (72 hrs.)	30.01	29.94±1.38	4.60	99.77
	638.42	599.80±13.62	2.27	93.25
Long Term stability (36 Days)	30.01	31.66±1.70	5.37	105.50

	638.42	613.30±27.44	4.47	96.07
Bench Top Stability (24 Hrs)	30.01	29.66±1.23	4.14	98.83
	638.42	598.69±15.50	2.59	93.78

## PHARMACOKINETIC STUDY

This validated method was used for the determination of Acamprosate in plasma samples to study the bioequivalence of Acamprosate calcium delayed-release tablets 333mg in the healthy adult human subject under fasting conditions. There was no adverse event reported during the study. No significant abnormalities were reported in the post-study physical examinations, vital sign, ECG and post clinical investigation report for all subjects.

The mean plasma concentration of analyte versus time profile was mentioned in the fig. 4.



**Figure 4: The mean plasma concentration of analyte versus time profile**

Plasma concentrations of the Acamprosate were within the calibration curve range. The pharmacokinetic parameters for the test and reference are mentioned in table 5-6. The LSM mean ratio of  $AUC_{(0-t)}$ ,  $AUC_{(0-\infty)}$  was higher than 90% as per the bioequivalence guidelines. The LSM ratio (Test / Reference) and 90 % confidence interval for the bioequivalence study was concluded between 80-125%. Therefore, it can be concluded that the two Acamprosate formulations (Test and Reference) analyzed are bioequivalent in the pharmacokinetics of fasting conditions.

**Table 5- Mean Pharmacokinetics Parameters**

Pharmacokinetics Parameters	Reference	Test
$AUC_{(0-t)}$	1557.03	1591.95
$AUC_{(0-\infty)}$	2720.21	2987.4
$C_{max}$	115.01	122.06
$K_{el}$	0.06	0.07
$T_{max}$	7.5	7

**Table 6- Pharmacokinetics Parameters**

<b>Pharmacokinetics Parameters</b>	AUC <sub>(0-t)</sub>	AUC <sub>(0-∞)</sub>	C <sub>max</sub>
Test / Reference Ratio	87.57	91.24	95.87

## CONCLUSION

This method is sensitive, selective, stable and reproducible. The analyte was compared in the presence of internal standard. The hybrid method (Precipitation and solid phase extraction) was used to extract the analyte from plasma samples. This method was validated over the concentration range of 10.01 ng/mL to 709.36 ng/mL as per the regulatory guidelines. This method was applied to study the pharmacokinetics parameters on healthy volunteers under fast conditions.

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