Drug Testing
and Analysis

Received: 22 September 2010 Revised: 8 November 2010 Accepted: 8 November 2010

(www.drugtestinganalysis.com) DOI 10.1002/dta.239

A LC-MS analysis of acamprosate from human plasma: pharmacokinetic application

**Chinmoy Ghosh,[a,b]* Vijay Jha,[a] Chandrakant P. Shinde[b]
and Bhaswat S. Chakraborty[a]**

A rapid and highly sensitive method for the determination of acamprosate (ACM), in human plasma using ESI-LC-MS/MS (electrospray ionization liquid chromatography tandem mass spectrometry) in negative ionization polarity in multiple reactions monitoring (MRM) mode was developed and validated. The procedure involves a simple protein precipitation step. Chromatographic separation was carried out on a Hypersil BDS C_{18} column (150 mm × 4.6 mm, 5 μm) with an isocratic mobile phase and a total run time of 2.5 min. The standard calibration curves were linear within the range of 7.04–702.20 ng/mL for ACM ($r \geq 0.990$). This study briefly describes the role of ion source design on matrix effects. ACM shows matrix effects in z-spray ionization source design, whereas it has no matrix effects in orthogonal spray ion source design. This method was successfully applied to a pharmacokinetic study after oral administration of acamprosate 333 mg tablet in Indian healthy male volunteers. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: acamprosate; ion source design; LC-MS/MS; matrix effect; pharmacokinetic

Introduction

Acamprosate (ACM) is a drug used for treating alcohol dependence. Acamprosate (ACM) (Figure 1) is a homotaurine derivative. It is a structural analogue of gamma amino butyric acid (GABA) and upper homologue of taurine. Its crossing through the blood brain barrier is facilitated by acetylation and calcium salification.[1] ACM has a very weak toxicity attributable to the ingested dose of calcium. ACM is known as a GABA agonist which shows pharmacological activity on direct and indirect tests of GABA activity. ACM shows high binding capacity with GABA receptors. ACM also shows beta-adrenergic and serotoninergic activity, probably due to its GABAergic activity. In experimental alcohology on different animal models (alcohol preferring or alcohol dependent), ACM induces a very clear and highly significant reduction of alcohol consumption. This effect is progressive and dose dependent, antagonized by bicuculine which is a GABA agonist. Moreover, ACM also reduces the intensity of the alcohol withdrawal syndrome.[1]

Mass spectrometry (MS) is a powerful qualitative and quantitative analytical technique that has been introduced into many clinical and research laboratories during the last few years. In the clinical laboratory, mass spectrometers are used to measure a wide range of clinically relevant analyte. When applied to biological samples, the power of MS lies in its selectivity towards the identification and quantification of compounds.

Developing and validating, a satisfactory and almost matrix effects free bioanalytical method is a preliminary step for any pharmacokinetic studies. However, a few bioanalytical methods have been developed for the determination of ACM concentration. Very few analytical methods have been reported for determining ACM levels in plasma and urine.[2,3] These methods are either based on high performance liquid chromatography (HPLC) with fluorescence or electrochemical detection[2,3] or gas chromatography (GC) with mass spectrometric detection.[4] The HPLC methods require multistep extractions and derivatization of acamprosate to produce a fluorescence-absorbing chromophore. The GC methods also require multistep extractions and derivatization of ACM with penta fluorobenzoyl chloride before injection into the GC/MS system.[5] Generally, in routine analysis, the derivatization step increases sample preparation time and the cost of the method. ACM is also highly hydrophilic, which renders it extremely difficult to extract from aqueous medium. Thus, complex (derivatization or long extraction procedures (multistep liquid-liquid extractions (LLE) or solid-phase extractions (SPE)) are required. Quantification of drugs in biological matrices by liquid chromatography tandem mass spectrometry (LC-MS/MS) is becoming more common, owing to the improved sensitivity and specificity of this technique.

There are a few LC-MS methods available[5–7] for the bioanalysis of ACM, but their lower limit of quantification (LLOQ) values are quite high. Moreover, none of these methods have discussed the matrix effects during MS analysis. These high LLOQ values in the published methods[5–7] may be attributed to matrix effects (ME), i.e. ion suppression. In the present research work, it is shown that ACM is prone to ME in z-spray ionization source (Waters Quattro Premier XE), whereas there is almost no ME in orthogonal spray ion source (Sciex API-4000, Applied Biosystem). So, the sensitivity level of the method has been improved significantly with LLOQ value approximately 7 ng/mL during analysis in API 4000 instrument.

This bioanalytical method was validated in human plasma which can be applied to bioequivalence and pharmacokinetic studies of ACM. The procedure requires a single-step protein precipitation

---

* *Correspondence to: Chinmoy Ghosh, Research Scientist, Contract Research Organization, Cadila Pharmaceuticals Limited, 1389, Trasad road, Dholka-387810 Dist – Ahmedabad, Gujrat, India. E-mail: chinmoy_ghosh@yahoo.com*

a *Bioanalytical Department, Cadila Pharmaceuticals Limited, 1389-Trasad road, Dholka, Gujarat, India*

b *School of Studies in Chemistry, Jiwaji University, Gwalior, M.P., India*

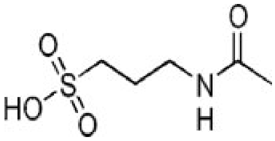

Figure 1. Structure of acamprosate.

without any derivatization. This method was employed in a pharmacokinetic study of a commercial 333 mg tablet of acamprosate in healthy Indian human volunteers.

Experimental

Chemicals and reagents

ACM (99.8%) and aceclofenac as internal standard (IS) were procured from Ind-Swift Laboratories Limited(Mohali, Punjab, India) and quality control department of Cadila Pharmaceuticals Limited (Ahmedabad, Gujarat, India), respectively. All reagents were of analytical reagent grade unless stated otherwise. Water used for the preparation of mobile phase and other solutions was collected from Milli Q_{PS} (Millipore, Billerica, Massachusetts USA) installed in the laboratory. HPLC-grade methanol, acetonitrile, and acetic acid were supplied by J. T. Baker (Massachusetts USA) and Finar Chemicals Limited (Ahmedabad, India), respectively. Ammonium acetate was purchased from Qualigens fine chemicals (Mumbai, India). Drug-free human K_2EDTA plasma was used during validation and study sample analysis was supplied by clinical unit of Cadila Pharmaceuticals Limited (Ahmedabad, India). Plasma was stored at $-30 \pm 5\,^{\circ}$C before sample preparation and analysis.

Standards and working solutions

An individual stock standard solution of ACM and the IS containing 1 mg/mL was prepared by dissolving pure compound in methanol. Intermediate and working solutions were prepared from corresponding stock solutions by diluting with a mixture of water: methanol, 50:50 *v/v*. Calibration standards were prepared in the range of 7.04 to 702.20 ng/mL using eight concentration levels each. Quality control samples of three different levels at low (20.88 ng/mL), medium (298.00 ng/mL), and high (595.96 ng/mL) were also prepared. All these stock solutions, calibration standards and quality control samples were stored at $6 \pm 2\,^{\circ}$C.

Analytical system

The plasma ACM concentrations were quantified using SCIEX API 4000 LC-MS/MS system (MDS Sciex, Ontario, Canada), equipped with an ESI interface used to generate negative ions $[M-H]^-$. The compounds were separated on a reversed phase column (Hypersil BDS C18, 150 × 4.6 mm ID, particle size 5 μ, Thermo Electron Corporation, UK), with an isocratic mobile phase consisting of 10 mM ammonium acetate (pH: 5.5) in water and acetonitrile at a ratio of 15 : 85, *v/v*. The mobile phase was eluted at 0.80 mL/min. Total analysis time of single injection was 2.5 min. Injection volume was 5 μL. Auto sampler rinsing volume was 500 μL. The column and auto sampler temperatures were maintained at 35° and 4 °C, respectively.

The optimized ion spray voltage and temperature were set at −4500 V and 450 °C. The typical ion source parameters, viz., declustering potential, collision energy, entrance potential and collision cell exit potential were −50, −10, −32, and −5 V for ACM and −30, −10, −35, and −10 V for the IS, respectively. Nitrogen gas was used as nebulizer gas, curtain gas, and collision-activated dissociation gas, which were set at 40, 12, and 7 psi, respectively. Quantification was performed by multiple reaction monitoring of the deprotonated precursor ion and the related product ion for ACM using the IS method with a peak area ratio and a linear least-squares regression curve with weighting factor of $1/x^2$. The mass transitions used for ACM and the IS were m/z 179.90 → 79.90 and m/z 353.70 → 74.90 respectively, with a dwell time of 400 ms per transition. Quadrupoles Q1 and Q3 were set on a unit resolution. The analytical data were processed by Analyst software (Version 1.4.2; Applied Biosystems Ontario, Canada).

Sample treatment

Protein precipitation extraction technique was used to extract ACM. Only 250 μL plasma sample was transferred to a ria vial for analysis. 20 μL of IS (10 μg/mL) was added into it, the sample was vortexed for 15 s. 1000 μL of acetonitrile was added into it to precipitate protein and was vortexed it for 1.5 min followed by centrifuge at 10000 RPM for 5 min. The supernatant is then injected (5 μL) to LC-MS/MS.

Clinical protocol

The bioequivalence study protocol and bioanalytical method presented in this paper were approved by the independent Medical Ethics Committee of Cadila Contract Research Organization, Ahmedabad, Gujarat, India. It was a open labelled, randomized, single and multiple dose pharmacokinetic study in which 02 subjects were administered a single dose of CAMPRAL Tablets (Acamprosate calcium 2 × 333 mg tablets) and 02 subjects were administered multiple (three doses) doses of CAMPRAL Tablets (Acamprosate calcium 2 × 333 mg tablets) along with 200 mL of drinking water after an overnight fasting of at least 10 h in each period with at least 10 days of the washout period between each administration. All subjects were healthy, adult, male, human Indian volunteers of Indian origin. In each period, a total of 24 blood samples were collected including a pre-dose sample prior to drug administration and after drug administration at 1.00, 2.00, 2.50, 3.00, 3.5, 04.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, 9.00, 12.00, 16.00, 24.00, 48.00, 72.00, 96.00, and 120.00 hours. The blood samples were immediately centrifuged at 2000 × g for 10 min at 4 °C, and the plasma samples were stored at −30 °C until LC-MS/MS analysis.

Results

Optimization of chromatographic condition and sample clean-up

The successful analysis of the analyte in biological fluids using LC-MS/MS relies on the optimization of chromatographic conditions, sample preparation techniques, chromatographic separation, and post-column detection, etc.[8,9]

ACM was first tuned by using Waters Quattro Premier XE mass spectrometer (Figure 2) which has a z-spray ionization source design. After optimization of tuning method for better selectivity and sensitivity, different types of columns and mobile phases were used. Length of the column varied from 50 mm to 150 mm, and the particle size varied from 3.5 μ to 5 μ. Columns of different

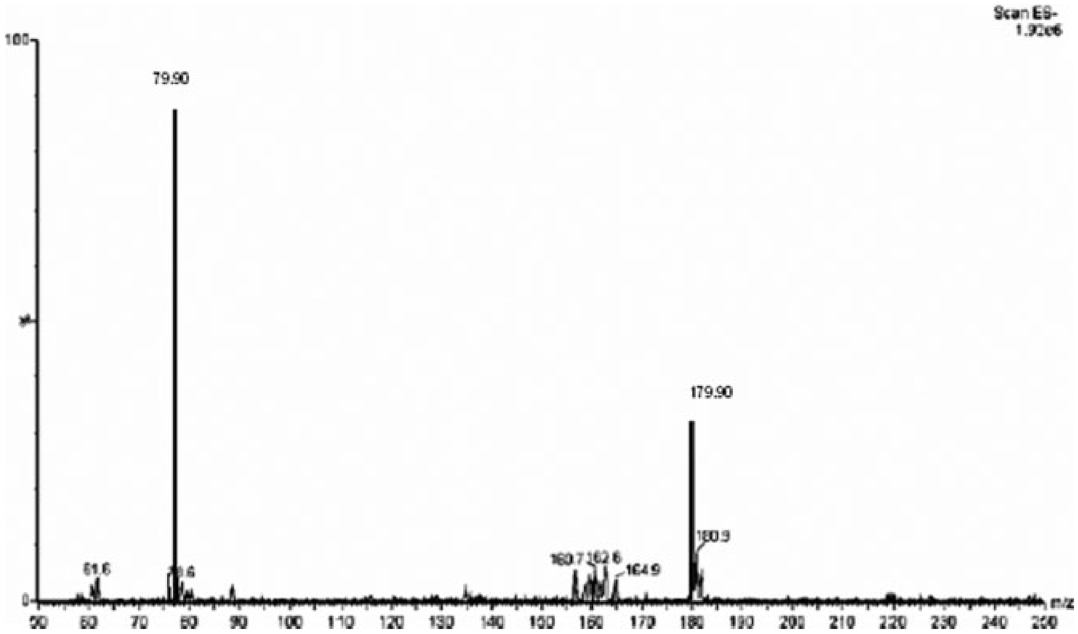

Figure 2. Negative ion electrospray mass spectrum obtained in product ion scan mode from standard sample of acamprosate.

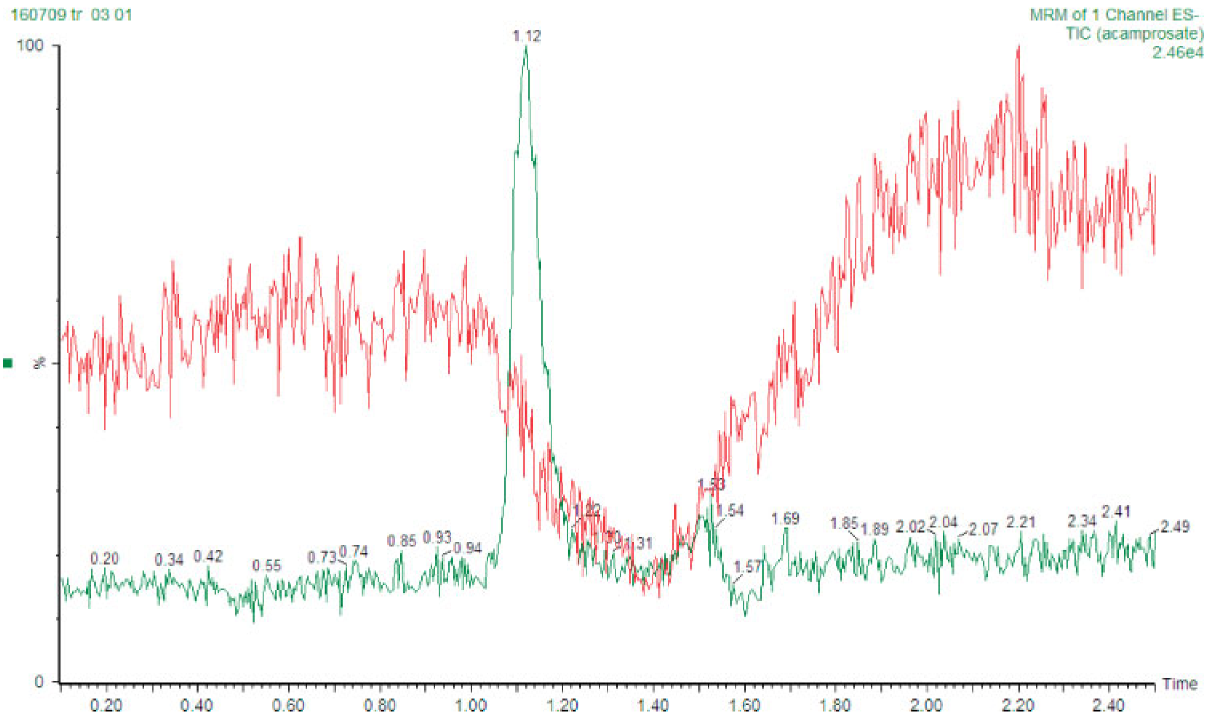

Figure 3. Matrix effects of acamprosate in Quattro Premier XE (ion suppression).

stationary phases such as C8, C18, cyano, etc., were used which showed some remarkable effect on interference and peak shape. Finally Hypersil BDS C18, 150 × 4.6 mm ID, analytical column of 5 µ particle size was selected for analysis. The influence of strength of the buffer, pH, and types of organic modifier on the signal intensities was also studied. Based on the peak intensity of the ACM and IS, a mixture of 10 mM ammonium acetate (pH 5.5) and acetonitrile (15:85 *v*/*v*) was selected as mobile phase in the final study with a flow rate of 0.800 mL/min. Initially a mixture of 90% acetonitrile: 10% of 0.6% Acetic acid (*v*/*v*) at a flow rate of 0.500 mL/min was tried but, very high proportion of organic phase led to improper elution leading to peak deformation. Therefore, the 85: 15 (*v*/*v*) organic phase to buffer was selected as optimum. Once the chromatographic parameters were finalized, sample extraction was optimized.

During sample extraction LLE, SPE, and protein precipitation techniques (PPT) were tried. Responses were observed in PPT method, whereas other extraction methods did not have any extraction recovery. Post-column T-split infusion of analyte solution during a chromatographic run of a plasma blank sample

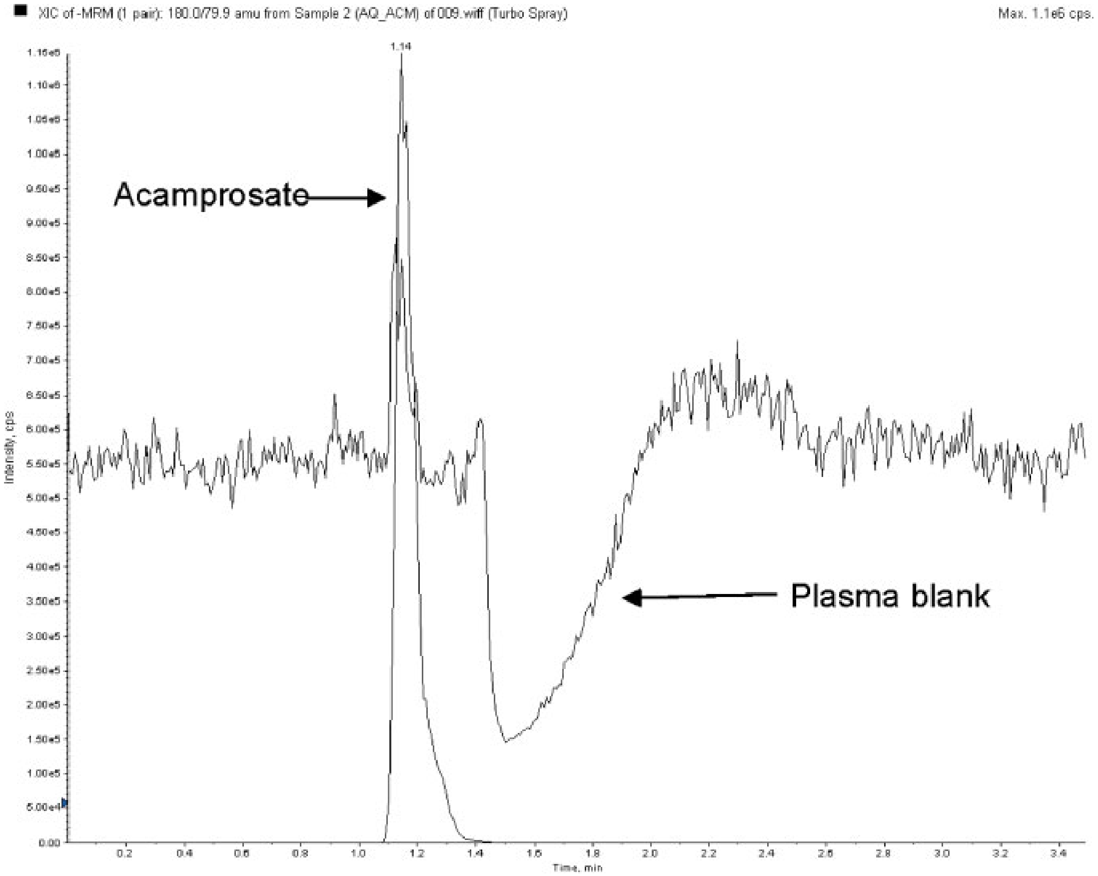

Figure 4. Matrix effects of acamprosate in API-4000 (ion enhancement).

was performed to check its ME. This experiment was performed by using two different ion sources. During the experiment, sharp ion suppression at the retention time (RT) of ACM (Figure 3) was observed in Waters Quattro Premier XE, whereas, there was very little ion enhancement at the RT of ACM (Figure 4) in API 4000, though the chromatographic conditions and extraction technique was same for both the instruments.

Finally ACM was analyzed by using API 4000 mass spectrometer with the abovementioned chromatographic condition and extraction technique.

Method validation

The validation parameters were specifically linearity, sensitivity, accuracy, precision, and matrix effects of the assay and the recovery and stability in human plasma, according to the US Food and Drug Administration (FDA) guidance for the validation of Bioanalytical methods.[10]

Selectivity was studied by comparing the chromatograms of eight different lots of plasma including one lipemic and one haemolyzed plasma obtained from different subjects, with the plasma samples having been spiked with ACM and the IS. Calibration curves were prepared by assaying standard plasma samples at ACM concentrations, ranging from 7.04 to 702.20 ng/mL.

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of ACM to the IS versus the nominal concentration (x) of ACM, respectively. The calibration curves were constructed by weighing ($1/x^2$) least-squares linear regression.

The LLOQ of ACM in human plasma was defined as the lowest concentration giving at least 20-fold, acceptable accuracy (80–120%), and sufficient precision (within 20%); this was verified by analysis of six replicates.

Intra- and inter-day accuracy and precision for this method was determined at three different concentration levels on three different days; on each day, six replicates were analyzed with independently prepared calibration curves. The percentage accuracy was expressed as (mean observed concentration)/(nominal concentration) ×100, and the precision was the relative standard deviation (RSD, %).

The stability of ACM was assessed by analyzing six replicate samples spiked with 20.88 and 595.96 ng/mL of ACM, respectively, under six conditions: after short-term stock solution storage for 7 h at room temperature; after short-term working solution storage for 7 h at room temperature; after five freeze-thaw cycles; after 6.5 h on bench top; after 46.5 h within the auto sampler. The concentrations obtained were compared with the nominal values of the QC samples.

Linearity

Linearity of calibration standards were assessed by subjecting the spiked concentrations and the respective peak areas using $1/X^2$ linear least-squares regression analysis. Linearity ranged from 7.04 to 702.20 ng/mL ($r > 0.990$). In aqueous solution, accuracy of all calibration standards was within 85–115%, except LLOQ where it was 80–120%.

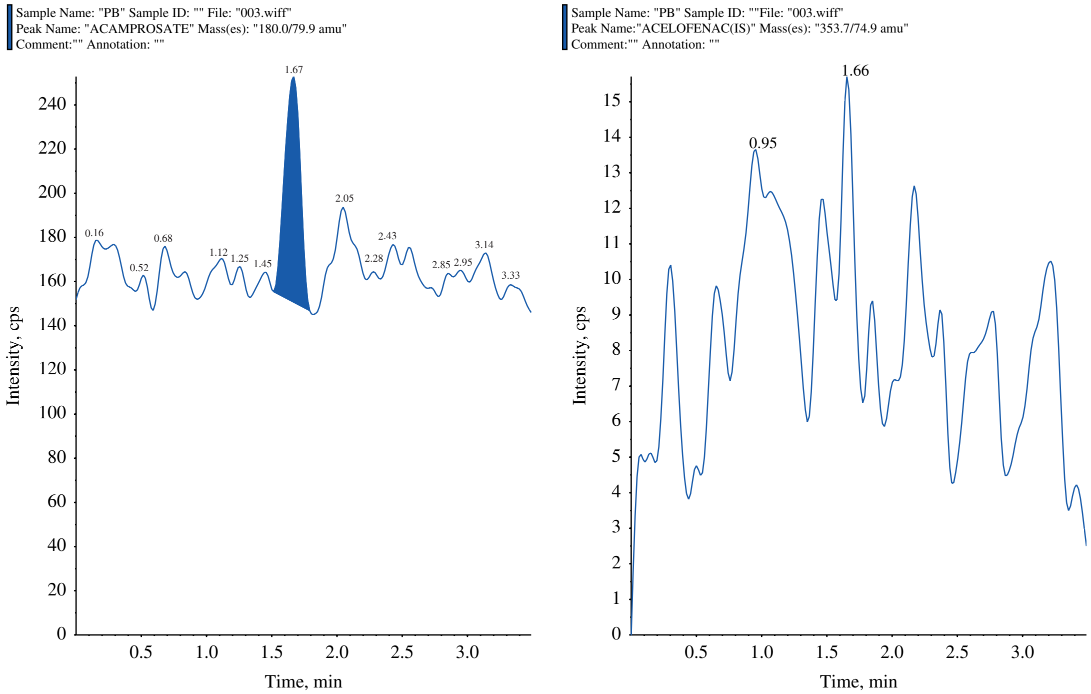

Figure 5. Representative chromatogram of plasma blank.

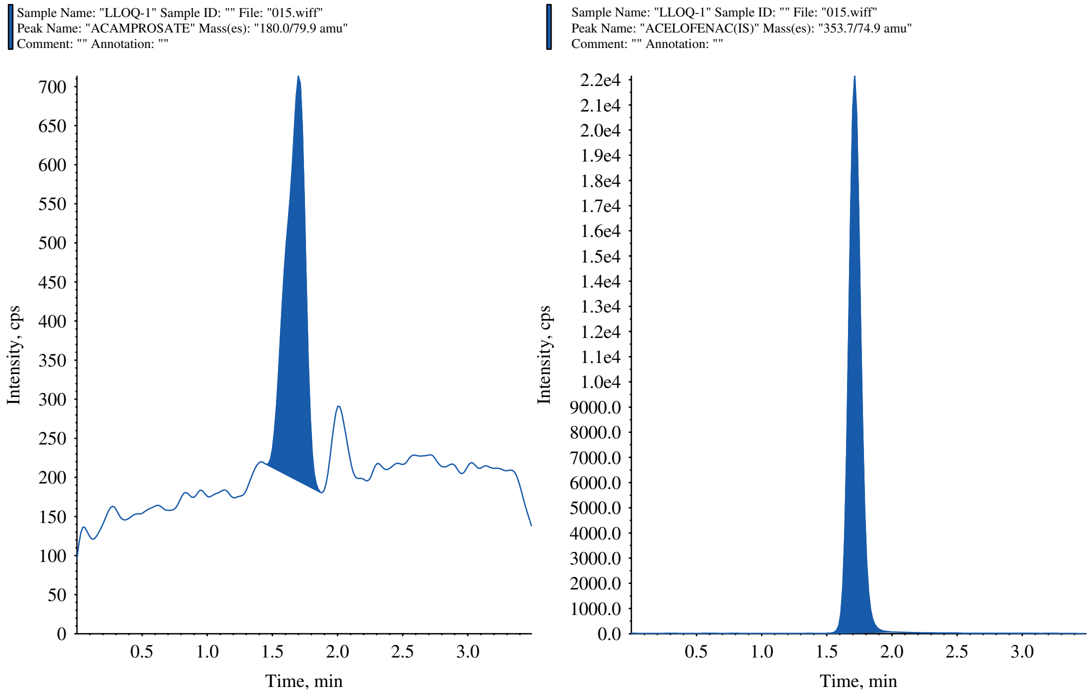

Figure 6. Representative chromatogram of LLOQ.

Specificity and selectivity

Selected blank human plasma sources were carried through the extraction procedure and chromatographed to determine the extent to which endogenous human plasma components may contribute to chromatographic interference with the ACM or IS. No significant interference was observed in eight different lots of blank human plasma samples including 1 haemolyzed and 1 lipemic plasma. In all plasma blanks (Figure 5), the response at the retention time of ACM was $<$ 20% of LLOQ response and at the retention time of IS, the response was $<$ 5% of mean IS response in LLOQ.

Sensitivity

Six LLOQ samples were injected along with the calibration curve in same range as used in precision and accuracy batch. Back calculated concentrations of LLOQ samples (Figure 6) were

Table 1 A. Inter- and intra-day accuracy and precision of ACM

	QC Levels	Mean accuracy	Mean Precision (% CV)
Day 1	LLOQ	95.03	6.07
	LQC	94.20	4.04
	MQC	105.28	2.60
	HQC	103.65	6.72
Day 2	LLOQ	100.00	6.70
	LQC	103.69	3.21
	MQC	111.70	5.00
	HQC	109.00	2.49
Day 3	LLOQ	112.22	5.68
	LQC	110.01	6.33
	MQC	107.86	5.05
	HQC	105.60	4.24

Table 1 B. Overall statistics of QC samples of precision and accuracy batches during validation of ACM

Analyte name	QC Levels	Nominal conc. (ng mL^{-1})	N	Mean conc. (ng mL^{-1})	SD(±)	%RSD	% Accuracy
ACM	LLOQ	7.04	18	7.21	0.668	9.26	102.41
	LQC	20.88	18	21.43	1.701	7.94	102.63
	MQC	298.00	18	322.69	15.622	4.84	108.29
	HQC	595.96	18	632.22	31.274	4.95	106.08

determined against calibration standards. For ACM % CV (% coefficients of variation) for LLOQ was 6.07 and % Nominal was 95.03.

Accuracy and precision

For the validation of the assay, QC samples were prepared at three concentration levels of low, medium, and high. Six replicates of each QC sample were analyzed together with a set of calibration standards. The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples in six replicate for three days. Obtained accuracy and precision (inter- and intra-day) are presented in Table 1A for ACM and the overall accuracy and precision are presented in Table 1B. The results showed that the analytical method was accurate, as the accuracy was within the acceptance limits of 100 ± 20% of the theoretical value at LLOQ and 100 ± 15% at all other concentration levels. The precision around the mean value was never greater than 15% at any of the concentration studied.

Recovery study

Recovery of ACM was evaluated by comparing mean analyte response of six extracted samples of low, medium, and high quality control samples to mean analyte response of six replicates injection of unextracted quality control samples. The mean recovery for LQC (low quality control), MQC (medium quality control), and HQC (high quality control) are 68.56, 66.24, and 67.23 respectively. % Coefficients of variation for the mean of all QC was found 1.73. The mean recovery for internal standard was 66.18.

Table 2. Summary of stability data of ACM

Experiment name	QC level	Mean accuracy	Mean Precision (%CV)	percent change	Stability Duration
Bench top	LQC	96.50	5.50	−6.93	06 h
	HQC	104.64	8.66	−4.00	
Freeze thaw	LQC	93.39	3.26	1.30	5 cycles
	HQC	93.47	4.44	2.30	
Auto sampler	LQC	105.03	13.56	−4.53	46 h
	HQC	107.50	2.85	1.80	

Matrix effects

Matrix effect was determined in terms of matrix factor (MF) to examine whether there was co-elution of analyte or IS with any matrix or its components. MF was determined in triplicate from six different plasma lots at LQC and HQC level by comparing with aqueous samples of LQC and HQC level. 10 µL of highest working calibration standard (WCS1) and 20 µL of working internal standard (WIS) were added to 1240 µL of processed blank plasma. These were injected along with the 18 replicates of aqueous LQC and HQC concentrations. The % CV of MF of eighteen LQCs and HQCs each was found to be 7.11 and 8.87 respectively. Whereas the overall %CV of MF of all QCs was 13.59 and the average MF of all LQCs and HQCs was 1.0082. The % CV of MF of IS was 1.85 and the MF of IS was 0.92. It showed that the method was free from any matrix interferences.

Haemolysis effects

For evaluation of potential interference of haemolyzed plasma sample with ACM and IS, six samples each of, LQC, MQC, and HQC were processed from haemolyzed plasma and analyzed with freshly processed calibration curve from normal plasma. The % CV for LQC was 2.01; for MQC was 5.18 and for HQC was 3.36. The % nominal for LQC was 104.26, MQC was 107.41 and for HQC was 110.01.

Stability studies

The stability of ACM and the IS were investigated in plasma during storage, during processing, after five freeze-thaw cycles, and in the final extract. Stability samples were compared with freshly processed calibration standards and QC samples. Analyte and IS were considered stable when the change of concentration is ±10% with respect to their initial concentration.

The % CV of ACM at LQC and HQC levels for freeze thaw stability, bench-top stability and auto sampler stability were 3.26, 5.50, and 13.56 and 8.66, 4.44, and 2.85, respectively. Summary of stability data were presented in Table 2.

Calibration curve parameters

The summary of calibration curve parameters was as follows. For ACM the mean slope and y-intercepts were 0.0046 (Range: 0.00311 to 0.00732) and 0.02058 (Range: 0.0147 to 0.0286), respectively. The mean correlation coefficient, r was 0.9973 (Range: 0.9942 to 0.9986).

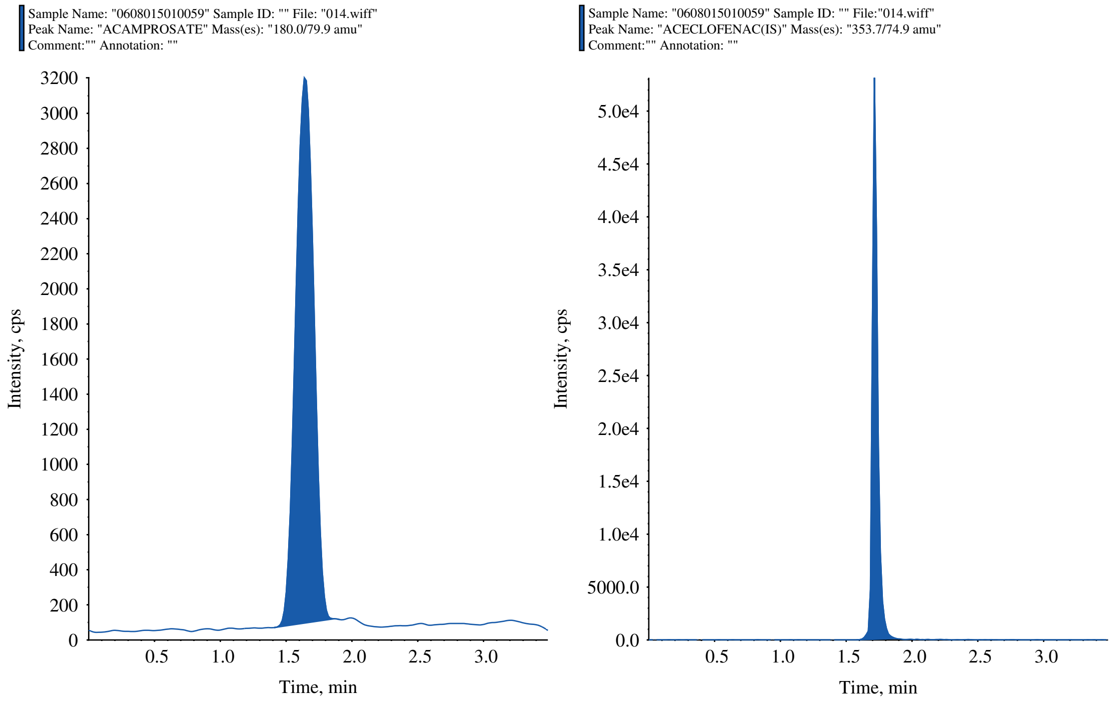

Figure 7. Representative chromatogram of real sample of acamprosate spiked with IS.

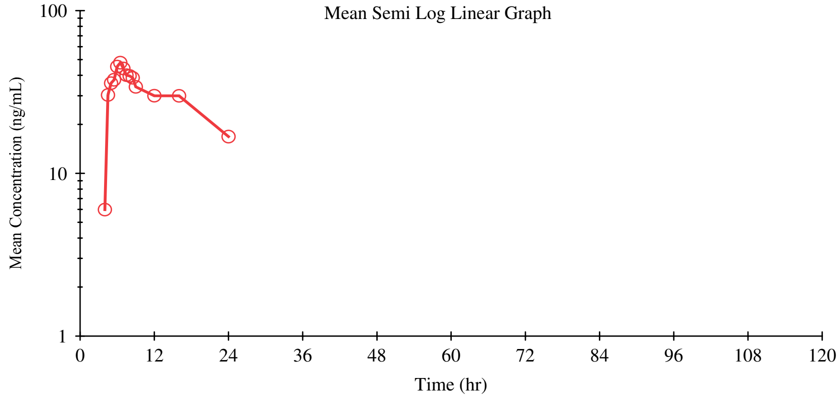

Figure 8. Graph of mean semi-log linear plasma concentration of single dose acamprosate tablet.

Application

The above-described fully validated method was applied to determine the concentration time profile following administration of ACM in healthy human volunteers. The chromatograms obtained from analysis of real samples are presented in Figure 7. After LC-MS/MS analysis the plasma concentration of ACM for all volunteers at times 0.00, 1.00, 2.00, 2.50, 3.00, 3.5, 04.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, 9.00, 12.00, 16.00, 24.00, 48.00, 72.00, 96.00, and 120.00 hours post dosing. For single and multiple dose reference products the C_{max} were 57.700 ± 2.920 ng/mL and 155.610 ± 6.810 ng/mL, AUC $_{0-t}$ for both doses were 594.330 ± 100.842 ng × h/mL and 2959.981 ± 1745.001 ng × h/mL, AUC $_{0-\propto}$ for both the doses were 889.136 ± 100.824 ng × h/mL and 4344.132 ± 3013.881 ng × h/mL, T_{max} for both the doses were 5.500 ± 1.000 h and 10.000 ± 6.000 h, $t_{1/2}$ for both the doses were 12.157 ± 0.317 h and 15.976 ± 12.294 h and K_{el} for both the doses were 0.057 ± 0.001 h^{-1} and 0.106 ± 0.082 h^{-1}. The concentration versus time profile for both single and multiple doses are presented in Figures 8 and 9, respectively.

Discussion

From the results obtained, it was observed that the method is rugged, which met all the criteria as per USFDA guideline. The above experiments also provide an idea about the method development approaches by using LC-MS/MS. It was also observed that ME in LC-MS/MS analysis from biological fluids not only depends on the sample extraction techniques and chromatographic conditions, but the design of ion source is also very important and key factor. So to remove or minimize the ME, differently designed ion sources should be taken into consideration. Moreover, this method requires only 0.250 mL of biological samples, owing to simple sample preparation and short run time (2.5 min), it allows high sample throughput.

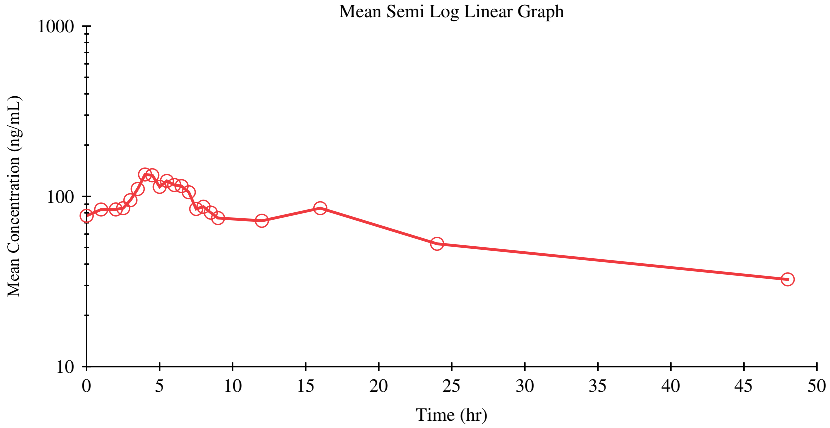

Figure 9. Graph of mean semi-log linear plasma concentration of multiple dose acamprosate tablet.

Conclusion

From the above experiments and discussions it can be concluded that it is the most sensitive method for determination of ACM with shortest analysis time. The method was successfully applied to a single dose 333 mg tablet bio equivalence study of ACM.

Acknowledgement

The authors would like to thank Mr. Ramesh Ahirand Ms Shweta Bhakti for his contribution and suggestions to the improvement of the method/manuscript described herein.

References

[1] B. Nalpas, H. Dabadie, P. Parrot, J. Paccalin, *Encephale.* **1990**, *16*, 175.
[2] C. Chabenat, P. Ladure, D. Blanc-Continsouza, F. Boismare, P. Boucly, *J. Chromatogr.* **1987**, *414*, 417.
[3] C. Chabenat, P. Ladure, N. Moore, P. Boucly, F. Boismare, *Arzneimittel-Forsch.* **1989**, *39*, 1413.
[4] J. Girault, P. Gobin, J. B. Fourtillan, *J Chromatogr-Biomed.* **1990**, *530*, 295.
[5] R. Yun-Seok, P. Jeong-Hwa, P. Seok, P. Chun-Woong, H. Jeong-Myung, J. Ki-Woo, L. Dong-Soo, P. Eun-Seok, *Arch. Pharm. Res.* **2008**, *31*, 1035.
[6] A. Hammarberg, O. Beck, S. Eksborg, N. Jayaram-Lindström, A. Lindefeldt, M. Andersson, L. Brundin, M. S. Reid, J. Franck, *Ther. Drug Monit.* **2010**, *32*, 489.
[7] F. Xu, Y. Qing, B. Shang, M. Liang, Y. Zou, G. Xu, *Arzneimittel-Forsch.* **2009**, *59*, 631.
[8] C. Ghosh, R. P. Singh, S. Inamdar, M. Mote, B. S. Chakraborty, *Chromatographia* **2009**, *69*, 1227.
[9] C. Ghosh, V. Jha, R. Ahir, S. Shah, C. P. Shinde, B. S. Chakraborty, *Drug Test. Analysis* **2010**, *2*, 284.
[10] Food and Drug Administration, *Guidance for Industry, Bioanalytical Method Validation*, CDER: Rockville, MD, **2001**.