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Development and validation of a selective and sensitive LC–MS/MS method for determination of cycloserine in human plasma: Application to bioequivalence study

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ABSTRACT

A selective and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for the determination of cycloserine in human plasma is developed using niacin as internal standard (IS). The analyte and IS were extracted from 500 μL of human plasma via solid phase extraction on Waters Oasis MCX cartridges. Chromatographic separation was achieved on a Peerless Basic C18 (100 mm × 4.6 mm, 3 μm) column under isocratic conditions. Detection of analyte and IS was done by tandem mass spectrometry, operating in positive ion and multiple reaction monitoring (MRM) acquisition mode. The protonated precursor to product ion transitions monitored for cycloserine and niacin were at *m/z* 103.1 → 75.0 and 124.1 → 80.1 respectively. The method was fully validated for its selectivity, interference check, sensitivity, carryover check, linearity, precision and accuracy, reinjection reproducibility, recovery, matrix effect, ion suppression/enhancement, stability and dilution integrity. The limit of detection (LOD) and lower limit of quantitation of the method were 0.0013 and 0.20 μg/mL respectively with a linear dynamic range of 0.20–30.00 μg/mL for cycloserine. The intra-batch and inter-batch precision (%CV) across six quality control levels was less than 8.0% for cycloserine. The method was successfully applied to a bioequivalence study of 250 mg cycloserine capsule formulation in 24 healthy Indian male subjects under fasting condition.

1. Introduction

Cycloserine (CYC, D-4-amino-3-isoxazolidinone), produced by streptomyces garyphalus and streptomyces orchidaceous is a broad-spectrum antibiotic, used primarily in the treatment of active pulmonary and extra-pulmonary tuberculosis [1–3]. Generally, it is administered in combination with other antitubercular agents for desired results. CYC is an analogue of the amino acid D-alanine and thus inhibits enzymes D-alanine racemase and D-alanine synthetase. It is more effective against Gram-positive than Gram-negative bacteria [4,5]. In addition, CYC has the ability to increase the level of the inhibitory neurotransmitter γ-aminobutyric acid *in vivo* and to inhibit the pyridoxal 5′-phosphate-dependent enzyme γ-aminobutyric acid aminotransferase *in vitro*. It is readily absorbed from the gastrointestinal tract after oral administration, with peak blood levels attained in 4–8 h [6].

Several methods are reported in literature to determine CYC in various drug products, but very few in biological matrices. El-Sayed et al. [7] determined CYC in capsules by a spectro-fluorimetric method by reaction with *p*-benzoquinine. CYC has also been determined in tablets by derivatization with 9-methoxyacridine in acetonitrile [8]. In another report, an n → π charge transfer complex of CYC with chloranil at pH 9.0 was determined by spectrophotometry [9]. A titrimetric method to estimate CYC from tablets has been proposed by Jayaram and Gowda [10]. A simple and sensitive second derivative UV spectrophotometric and HPLC with fluorometric detection methods were developed for cycloserine based on derivatization with 9-chloro-10-methyl acridinium triflate by Yoo et al. [11]. The HPLC assay was applied to the determination of CYC in spiked urine samples. A gradient liquid chromatographic method

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has been described for the determination of CYC and its degradation impurities in commercial drug substance (raw material) and a drug product (capsules) [12]. A capillary electrophoresis-mass spectrometry method has been utilized to estimate enantiomeric purity of D-CYC employing crown ether as a chiral selector [13]. Very recently, a sensitive, stability indicating method for L-CYC enantiomer impurity determination in D-CYC drug substance by reversed phase HPLC has been proposed [14]. CYC along with other antimycobacterial drugs like amikacin, kanamycin, ofloxacin, *p*-salicyclic acid, prothionamide, pyrazimamide and thambutol have been determined in serum samples by reversed phase HPLC, GLC and fluorescent polarization immunoassay [15].

An ion-pair, reversed phase HPLC assay was developed for the simultaneous determination of CYC and its prodrug acetylacetonylcycloserine (ACS) in human plasma and urine [16]. Detection of ACS was carried out by UV, while CYC was determined by post-column derivatization with *o*-phthalaldehyde and subsequent fluorescence measurement. ACS and CYC were quantified in plasma in the concentration range of 0.3–7.5 μg/mL and 0.3–15.0 μg/mL respectively. Both the analytes gave a linear response in the range of 2–100 μg/mL in urine. David et al. [17] described a method to determine CYC in human plasma by HPLC with fluorescence detection using derivatization with *p*-benzoquinone. They gave an insight to interpret the mechanism of derivatization reaction in which more than two derivatives are possible. All four derivatives formed were chromatographically separated on XDB C-18 (double end capped octadecyl silica gel) column in 15 min under isocratic conditions. The LOD and LOQ values were estimated to be 10 and 33.3 ng/mL respectively.

So far majority of the methods developed for CYC are based on derivatization reaction for fluorescence detection, however, to the best of our knowledge there is no report on the use of mass spectrometry detection to estimate CYC in human plasma. Thus, in the present study a sensitive, selective and rapid LC–MS/MS method has been proposed for the determination of CYC in human plasma. The analyte was extracted via simple SPE procedure and separated on a Peerless Basic C18 column under isocratic conditions. The method was extensively validated as per the United States Food and Drug Administration (USFDA) guidelines and is rugged and adequately sensitive for routine subject sample analysis. The method was successfully applied to a bioequivalence study of 250 mg cycloserine capsule formulation in 24 healthy Indian male subjects under fasting condition.

2. Experimental

2.1. Chemicals and materials

Reference standard material of cycloserine (98.3%) was procured from a Simson Pharma (Mumbai, India), while niacin (IS, 99.4%) was procured from Varda Biotech (P) Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were procured from S.D. Fine Chemicals Ltd. (Mumbai, India). Analytical grade reagent formic acid (90.0%) and ammonia solution (30.0%) were obtained from S.D. Fine Chemicals Ltd. (Mumbai, India). Water used for LC–MS/MS was prepared using Milli Q water purification system from Millipore (Bangalore, India). Oasis MCX (1 cm^3, 30 mg) extraction cartridges were from Waters Corporation (Milford, MA, USA). Control buffered (Na–Heparin) human plasma was procured from Clinical Department, BA Research India Limited (Ahmedabad, India) and was stored at −20 °C. Centrifuge was of Eppendorf 5810 (Hamburg, Germany).

Elution solution for SPE: 5.0% ammonia solution in methanol.

Reconstitution solution: 0.005% formic acid in deionized water:acetonitrile (20:80, *v/v*).

2.2. LC–MS/MS instrumentation and conditions

The liquid chromatography system from Shimadzu (Kyoto, Japan) consisted of a LC-10ADvp pump, an autosampler (SIL-HTc) and an on-line degasser (DGU-14A). Chromatographic column used was Peerless Basic C18 (100 mm length × 4.6 mm inner diameter, with 3.0 μm particle size) from Chromatopak Analytical Instrumentation Pvt. Ltd. (Mumbai, India). The mobile phase consisted of acetonitrile–0.005% formic acid in deionized water (70:30, *v/v*). Separation of analyte and IS was performed under isocratic condition at a flow rate of 0.8 mL/min. The auto sampler temperature was maintained at 4 °C and the injection volume was kept at 2.0 μL. The total LC run time was 5.0 min. Ionization and detection of analyte and IS was performed on a triple quadrupole mass spectrometer, API-3000 equipped with Turbo Ion spray®, from MDS SCIEX (Toronto, Canada) operating in the positive ion mode. Quantitation was done using MRM mode to monitor protonated precursor → product ion transition of *m/z* 103.1 → 75.0 for CYC and 124.1 → 80.1 for IS (Fig. 1a and b). All the parameters of LC and MS were controlled by Analyst software version 1.4.2.

For CYC and IS the source dependant parameters maintained were Gas 1(Nebulizer gas): 10 psi, ion spray voltage (ISV): 5000 V, turbo heater temperature (TEM): 500 °C, entrance potential (EP): 10 V, collision activation dissociation (CAD): 4 psi, curtain gas (CUR): 14 psi. The compound dependent parameters like declustering potential (DP), focusing potential (FP) collision energy (CE) and cell exit potential (CXP) were optimized at 30, 150, 10 and 10 V for CYC and 37, 300, 36 and 30 V for IS respectively. Quadrupole 1 and quadrupole 3 were maintained at unit resolution. Dwell time set was 1200 ms and 300 ms for CYC and IS respectively.

2.3. Preparation of standard stock and plasma samples

The CYC standard stock solution of 3000 μg/mL was prepared by dissolving requisite amount in deionized water. This was further diluted in deionized water to get an intermediate solution of 600.0 μg/mL. The working solutions of CYC for spiking plasma calibration and quality control samples were subsequently prepared using the standard and intermediate stock solutions in deionized water. The IS stock solutions of 1000 μg/mL was prepared by dissolving requisite amount of niacin in methanol. IS working solution (15.00 μg/mL) was prepared using the stock of 1000 μg/mL in deionized water. All the above solutions were stored at 4 °C until use. Drug free plasma, i.e. control (blank) plasma was withdrawn from the deep freezer and allowed to get completely thawed before use. The calibration standards (CS) and quality control (QC) samples (LLOQ QC, lower limit of quantitation quality control; LQC, low quality control; MQC-1 and MQC-2, medium quality control; HQC, high quality control; ULOQ QC, upper limit of quantitation quality control) were prepared by spiking blank plasma with respective working solutions (5% of total volume of plasma). CSs were made at 0.20, 0.40, 0.80, 2.00, 4.00, 8.00, 12.00, 16.00, 24.00 and 30.00 μg/mL for CYC. QCs were prepared at 0.20 μg/mL (LLOQ), 0.60 μg/mL (LQC), 3.70 μg/mL (MQC-2), 10.00 μg/mL (MQC-1), 22.50 μg/mL (HQC) and 30.00 μg/mL (ULOQ) concentrations. The spiked plasma samples at all the levels were stored at −20 °C for validation and subject sample analysis.

2.4. Protocol for sample preparation

Prior to analysis, spiked plasma samples were thawed and allowed to equilibrate at room temperature. The samples were adequately vortexed using a vortexer before pipetting. Aliquots of 500 μL plasma solution containing 25 μL of working solution of CYC and 475 μL blank plasma were transferred into ria vials, 100 μL working solution of IS (15.00 μg/mL) was added and

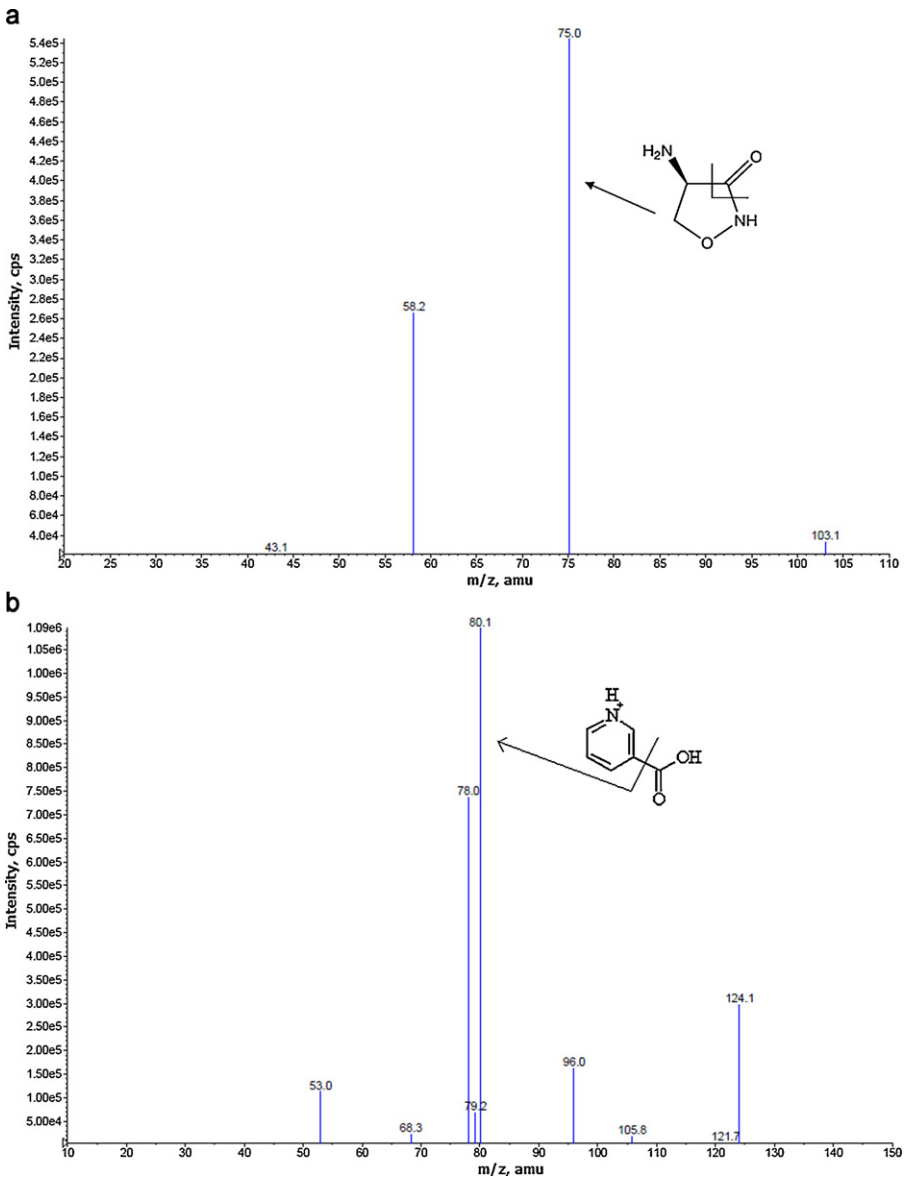

Fig. 1. Product ion mass spectra of (a) cycloserine (m/z 103.1 → 75.0, scan range 20–110 amu) and (b) niacin (IS, m/z 124.1 → 80.1, scan range 10–150 amu) in positive ion mode.

vortexed to mix. To the same ria vials, 100 μL of 2% (*v/v*) formic acid solution was added and vortexed again. Prior to loading plasma samples, SPE cartridges were pre-washed with 1.0 mL of methanol, followed by 1.0 mL of deionized water and centrifuged for 1 min at 1811 × *g*. Plasma samples were then applied to these conditioned cartridges and centrifugation was done at 1811 × *g* for 2 min. Further, washing was done with 1.0 mL of 2% (*v/v*) formic acid and 2 × 1.0 mL of methanol, followed by centrifugation at 1811 × *g* after each step. Elution was carried out with 1.0 mL of elution solution followed by centrifugation for 1 min at 1811 × *g*. The eluate was evaporated to dryness under gentle stream of nitrogen (15 psi) at 40 °C. The residue was taken up in 500 μL of reconstitution solution and 2.0 μL was used for injection in LC–MS/MS, in partial loop mode.

2.5. Methodology for validation

A thorough and complete method validation of CYC in human plasma was done following the USFDA guidelines [18]. The method was validated for selectivity, sensitivity, interference check, carryover check, linearity, precision and accuracy, reinjection reproducibility, recovery, ion suppression/enhancement, matrix effect, LOD check, stability and dilution integrity.

Test for selectivity was carried out in 14 different lots of blank human plasma including haemolysed and lipemic plasma collected with Na–heparin as an anticoagulant. From each of these 14 different lots, two replicates each of 475 μL were spiked with 25 μL of deionized water. In the first set, the blank human plasma was directly injected after extraction (without analyte and IS), while the other set was spiked with only IS before extraction (total 28 samples). Further, one system suitability sample (SSS) at CS-2 (0.40 μg/mL) concentration and two replicates of LLOQ concentration (CS-1) were prepared by spiking 475 μL blank human plasma with 25 μL of respective working aqueous standards of CYC. The blank human plasma used for spiking of SSS and LLOQ were chosen from one of these 14 lots of plasma. The acceptance criterion requires that at least 90% of selectivity samples should be free from any interference at the retention time of analyte and IS.

The interference due to commonly used medications in human volunteers was done for acetaminophen, aspirin, caffeine, cetrizine,

chlorpheniramine maleate, ibuprofen and pseudoephedrine. Their stock solutions (100.0 μg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (20.00 μg/mL) were prepared in deionized water. 25 μL of 20.00 μg/mL working solution of each commonly used medication were spiked in 475 μL of blank human plasma into ria vials and vortex to mix. These plasma samples were processed without IS by the proposed extraction protocol and 2.0 μL was injected to check any possible interference at the retention time of analyte and IS. Also, the possible interference due to CYC at the retention time of IS was checked during interference check. Carry over experiment was performed to verify any carryover of analyte, which may reflect in subsequent runs. The design of the study comprised of the following sequence of injections i.e. double blank plasma sample → two samples of LLOQ → double blank plasma → ULOQ sample → double blank plasma → ULOQ sample → double blank plasma, to check for any interference due to carry over.

The linearity of the method was determined by analysis of six calibration curves containing ten non-zero concentrations. The area ratio response for CYC/IS obtained from multiple reaction monitoring was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression which was finalized during pre-method validation. A correlation coefficient (r^2) value > 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least ten times more than that of drug free (blank) extracted plasma.

Intra-batch and inter-batch (on three consecutive validation days) accuracy and precision were evaluated at six QC levels (LLOQ QC, LQC, MQC-2, MQC-1, HQC and ULOQ QC) in six replicates for CYC. The deviation (%CV) at each concentration level from the nominal concentration was expected to be within ±15%. Similarly, the mean accuracy should not vary by ±15% except for the LLOQ where it can be ±20% of the nominal concentration. Reinjection reproducibility was performed by re-injecting one validation batch. The precision and accuracy for all the quality control samples were checked. Also, 2/3 quality control samples should meet the criteria of ±15% of nominal concentration.

The relative recovery, matrix effect and process efficiency were assessed as recommended by Matuszewski et al. [19]. All three parameters were evaluated at HQC, MQC-1, MQC-2 and LQC levels in six replicates. Relative recovery (RE) was calculated by comparing the mean area response of pre-spiked samples (spiked before extraction) to that of extracts with post-spiked samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions (in mobile phase). The overall 'process efficiency' (%PE) was calculated as (ME × RE)/100. Further, the effect of plasma matrix (relative matrix effect) on analyte quantification was also checked in eight different batches/lots of Na–heparin plasma including haemolysed and lipemic plasma. From each batch, four samples at LQC and HQC levels was prepared (spiked after extraction) and checked for the % accuracy and precision (%CV). The deviation of the standards and QCs should not be more than ±15%. Matrix ion suppression effects on the MRM LC–MS/MS sensitivity were evaluated by the post column analyte infusion experiment. A standard solution containing 30.00 μg/mL of CYC and 15.00 μg/mL of IS in mobile phase was infused post column via a 'T' connector into the mobile phase at 5.0 μL/min employing Harvard infusion pump. Aliquots of 2.0 μL of extracted 30.00 μg/mL sample and blank (without IS) sample were then injected and MRM LC–MS/MS chromatograms were acquired for CYC and IS. Any dip in the baseline upon injection of double blank plasma would indicate ion suppression, while a peak at the retention time of CYC and IS indicates ion enhancement.

All stability results were evaluated by measuring the area response (CYC/IS) of stability samples against freshly prepared comparison standards at LQC and HQC levels. Stock solutions of CYC and IS were checked for short term stability at room temperature and long term stability at 4 °C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Bench top stability, processed sample stability at room temperature and at refrigerated temperature (4 °C), freeze thaw stability and long term stability at −20 °C were performed at LQC and HQC levels using six replicates at each level. To meet the acceptance criteria the %CV and % accuracy should be within ±15%. Also, at least 2/3 quality control samples should meet the criteria of ±15% of nominal concentration.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 5 times the ULOQ concentration i.e. 150.0 μg/mL and at HQC level for CYC. Six replicate samples each of 1/10 of 5 × ULOQ (15.00 μg/mL) and 1/10 of HQC (2.25 μg/mL) concentration were prepared and their concentrations were calculated, by applying the dilution factor of 10 against the freshly prepared calibration curve for CYC.

2.6. Bioequivalence study design

The design of study comprised of "An open label, randomized, two period, two treatment, two sequence, balanced, single dose, crossover, comparative evaluation of relative oral bioavailability of test (250 mg cycloserine capsule of an Indian company) and reference formulation (SEROMYCIN®, 250 mg cycloserine capsule from Eli Lilly and Company, Indianapolis, USA) in 24 healthy Indian human subjects under fasting conditions". All the subjects were informed of the aim and risk involved in the study and written consent were obtained. The inclusion criteria for volunteer selection was based on the age (18years or above), body mass index (between 18.5 and 30.0 kg/height^2), general physical examination, electrocardiogram and laboratory tests like hematology, blood chemistry, urine examination and immunological tests. The exclusion criteria included allergic responses to CYC, volunteers with history of alcoholism, smokers and having a disease which may compromise the haemopoietic, gastrointestinal, renal, hepatic, cardiovascular, respiratory, central nervous system, diabetes, psychosis or any other body system. The work was approved and subject to review by Institutional Ethics Committee, an independent body comprising of eight members which includes a lawyer, medical doctors, social workers, pharmacologists and academicians. The procedures followed while dealing with human subjects were based on International Conference on Harmonization, E6 Good Clinical Practice (ICH, E6 GCP) guidelines [20]. Blood samples were collected in vacutainers containing Na–heparin anticoagulant before (0.0 h) and at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0, 30.0, 36.0, 48.0 h of administration of drug. Blood samples were centrifuged at 1811 × *g* at 4 °C for 15 min and plasma was separated, stored at −20 °C until use. The plasma concentration-time profiles obtained from the experimental subjects were analyzed by non-compartmental analysis using WinNonlin® professional software (version: 5.2.1) from Pharsight Corporation (Mountain View, CA, USA). An incurred sample re-analysis (ISR) was also conducted by computerized random selection of 92 subject samples (10% of total study samples analyzed) near C_{max} and the elimination phase. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than ±20% [21].

3. Results and discussion

3.1. Method development

As the literature reveals very few methods are reported, mainly based on HPLC with fluorescence detection to determine CYC in biological samples. Thus, in the present study method development was initiated to realize a rugged, sensitive and specific turbo ion spray LC–MS/MS method to quantify CYC in human plasma. To accomplish this aim it was imperative to have an efficient extraction procedure, with a short chromatographic run time. Also, the sensitivity should be adequate enough to monitor at least five half lives of CYC concentration with good accuracy and precision for subject samples. The tuning of MS parameters was carried out in positive as well as negative ionization modes for CYC and niacin (IS) using 50.0 ng/mL tuning solution. The response observed was much higher in positive ionization mode for both the drugs compared to the negative mode due to their basic nature with low background noise, resulting in higher sensitivity. The analyte and IS gave predominant singly charged protonated precursor $[M+H]^+$ ions at *m/z* of 103.1 and 124.1 for CYC and IS respectively in Q1 full scan spectra. Further, fragmentation was initiated using sufficient nitrogen for CAD and by applying 30.0 V collision energy to break the precursor ions. The most abundant ions found in the product ion mass spectra were at *m/z* 75.0 and 80.1 at 10.0 V and 36.0 V collision energy for CYC and IS respectively. To attain an ideal Taylor cone and a better impact on spectral response, nebulizer gas pressure was optimized at 10.0 psi. Fine tuning of nebulizer gas and CAD gas was done to get a consistent and stable response. Ion spray voltage and temperature did not have any significant effect on analyte response and hence were maintained at 5000 V and 500.0 °C respectively. A dwell time of 1200 ms and 300 ms was adequate and no cross talk was observed between the MRMs of analytes.

The chromatographic conditions were aimed to achieve an efficient separation and resolution from endogenous peaks. Also, the response should be adequate with sharp peak shape and a short run time per analysis for CYC and IS. This included mobile phase selection, flow rate, column type and injection volume. Different volume ratios (10:90, 20:80 and 30:70, *v/v*) of water–methanol and water–acetonitrile combinations were tried as mobile phase, along with formic acid (0.01–0.005%), ammonium trifluoroacetate and ammonium acetate buffers (2–20 mM) in varying strength on Hypurity C8 (100 mm × 4.6 mm, 5 μm), Hypurity cyano (50 mm × 4.6 mm, 5 μm), Beta basic cyano (100 mm × 2.1 mm, 5 μm), BDS Hypersil C18 (100 mm × 4.6 mm, 3 μm) and Peerless Basic C18 (100 mm × 4.6 mm, 3 μm). In addition, the effect of flow rate was also studied from 0.5 to 1.0 mL/min, which was also responsible for acceptable chromatographic peak shapes. The use of Peerless Basic C18 (100 mm × 4.6 mm, 3 μm) chromatography column helped in the separation and elution of both the analytes in a short time. An optimum mobile phase for their separation was very critical as they had similar retention behaviour and retention time. The mobile phase consisting of 0.005% formic acid solution: acetonitrile (30:70, *v/v*) was found most appropriate for faster elution, improved efficiency and peak shape. The retention time for CYC and IS was 1.19 and 1.28 min respectively at a flow rate of 0.8 mL/min. Under the optimized experimental conditions, the dead time for the column was 0.98 min. The maximum on-column loading (at ULOQ) of CYC per sample injection was 60 ng. Ideally, a deuterated analogue should be the first-choice internal standard, but due to its unavailability, a general IS was used to minimize analytical variation due to solvent evaporation, integrity of the column and ionization efficiency. Three drug compounds were tested, namely imipramine, levetiracetam and niacin for suitable selection of IS. Niacin, used as an IS in the present study had similar chromatographic behaviour and was easily separated and eluted along with the analyte. There was no effect of IS on analyte recovery, sensitivity or ion suppression. Also, the validation results obtained from this LC–MS/MS methodology encouraged its selection as an IS for the present study.

Quantitative extraction of analyte and IS was difficult due to ion suppression and selectivity issues. Thus, the anticoagulant effect was checked initially during plasma extraction to have adequate selectivity, peak shape and desired response using K3EDTA, K2EDTA and Na–heparin. Nevertheless, best results were obtained with Na–heparin and were used in the present study. Previous studies have employed ultrafiltration [16] and protein precipitation (PP) with ethanol [17] for sample preparation from human plasma. Initially, PP was tried using methanol, ethanol and acetonitrile as precipitating agents but the response was insufficient. Liquid–liquid extraction (LLE) was carried out with dichloromethane, diethyl ether, ethyl acetate and methyl-*tert*-butyl ether, however, the response was inconsistent with some matrix interference, especially at LLOQ level. Hence, SPE was tried on Oasis MCX cartridge to extract both the drugs by adding 2% (*v/v*) formic acid solution to break the drug protein binding. Significant efforts were aimed at improving the method ruggedness during the SPE and to get cleaner samples compared to PP and LLE. The recoveries obtained were quantitative for IS, but were low for CYC (47.7%). The possible reason for this low recovery of the analyte could be due to the additional washing step with 100% methanol. According to the USFDA guidelines [18], the recovery need not be 100%, but the extent of recovery of an analyte should be consistent and reproducible at each QC levels. Multiple extractions to further increase in recovery were deliberately avoided as it was time consuming and was less suitable for high throughput analysis. Also the validation results and subject sample reanalysis support this extraction methodology and hence were accepted in the present study.

3.2. Selectivity, interference and carryover check

The aim of performing selectivity check with 14 different plasma samples was to determine the extent to which endogenous plasma components might contribute to the interference at the retention time of analyte and the IS and thus, ensure the authenticity of the results for study sample analysis. All samples studied were found free from any endogenous interference. Fig. 2(a–d) demonstrates the selectivity results with the chromatograms of double blank plasma (without IS), blank plasma (with IS), peak response of CYC at LLOQ (0.20 μg/mL) concentration and the real subject sample chromatogram for CYC at 0.5 h after oral administration of 250 mg capsule formulation.

No interference was observed for commonly used medications like acetaminophen, aspirin, caffeine, cetrizine, chlorpheniramine maleate, ibuprofen and pseudoephedrine; this is evident from the ion chromatograms at LLOQ level and real subject sample chromatograms of CYC at C_{max} respectively. Carry-over evaluation was performed to ensure that it does not affect the accuracy and precision of the proposed method. Almost negligible area (less than 1% of LLOQ area) was observed in double blank plasma run after ULOQ, which suggests no carry-over of the analyte in subsequent runs. Moreover, no ghost peaks appears during the analysis of blank samples.

3.3. Linearity, sensitivity, accuracy and precision

All six calibration curves were linear over the concentration range of 0.20–30.00 μg/mL with correlation coefficient $r \geq 0.9987$. A straight-line fit was made through the data points by least square regression analysis to give the mean linear equation $y = 0.3823x - 0.0171$ where y is the peak area ratio of the analyte/IS

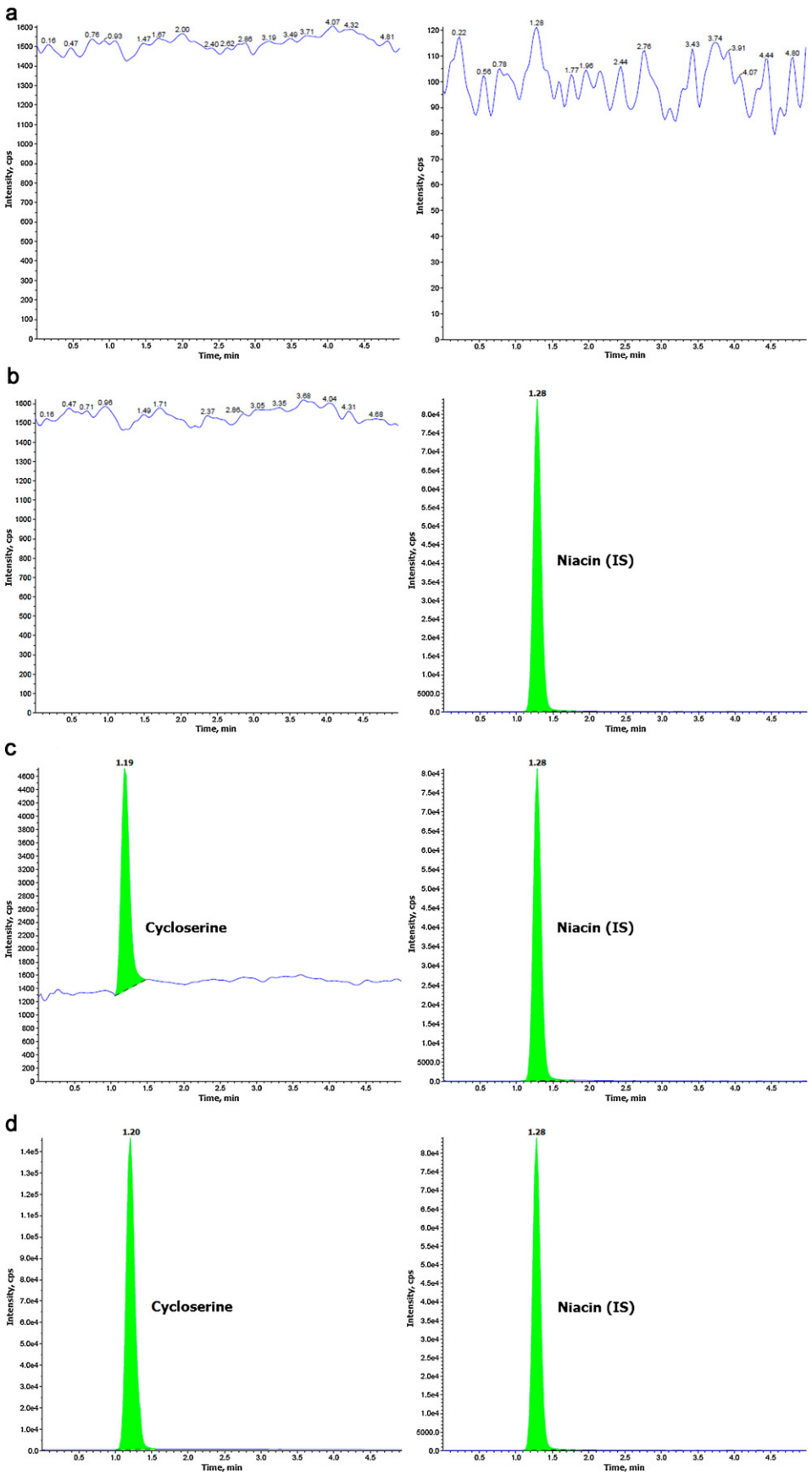

Fig. 2. MRM ion-chromatograms of cycloserine (*m/z* 103.1 → 75.0) and niacin (IS, *m/z* 124.1 → 80.1) in (a) double blank plasma (without analyte and IS), (b) blank plasma with IS, (c) cycloserine at LLOQ and IS (d) real subject sample at C_{max} after administration of 250 mg dose of cycloserine.

Table 1
Intra-batch and inter-batch accuracy and precision for cycloserine.

QC ID	Conc. added (μg/mL)	Intra-batch				Inter-batch			
		n	Mean Conc. found (μg/mL)[a]	Accuracy (%)	CV (%)	*n*	Mean Conc. found (μg/mL)[b]	Accuracy (%)	CV (%)
LLOQ	0.20	6	0.20	100.5	3.7	18	0.20	100.2	3.6
LQC	0.60	6	0.59	98.3	2.1	18	0.56	93.3	5.3
MQC-2	3.70	6	3.56	96.2	1.7	18	3.49	94.3	4.4
MQC-1	10.00	6	9.61	96.1	1.1	18	9.46	94.6	5.8
HQC	22.50	6	21.66	96.3	3.2	18	22.56	100.3	7.8
ULOQ	30.00	6	28.41	94.7	6.8	18	29.49	98.3	5.4

n: total number of observations.
CV: coefficient of variation.
[a] Mean of six replicate observations at each concentration.
[b] Mean of eighteen replicate observations over three different analytical runs.

Table 2
Absolute matrix effect, relative recovery and process efficiency for cycloserine.

A[a] (%CV)	*B*[b] (%CV)	*C*[c] (%CV)	Absolute matrix effect (% ME)[d]	Relative recovery (% RE)[e]	Process efficiency (% PE)[f]
LQC					
107, 574(3.57)	110,801 (2.37)	47,222 (2.93)	103.0	42.6	43.9
MQC-2					
689, 542(2.71)	717,124 (3.32)	335,755 (3.51)	104.0	46.8	48.7
MQC-1					
1, 918, 640(1.83)	1,937,826 (1.65)	983,706 (4.82)	101.0	50.8	51.3
HQC					
4, 636, 640(1.30)	4,729,373 (2.26)	2,387,445 (1.55)	102.0	50.5	51.5

CV: coefficient of variation.
[a] Mean area response of six replicate samples prepared in mobile phase (neat samples).
[b] Mean area response of six replicate samples prepared by spiking in extracted blank plasma.
[c] Mean area response of six replicate samples prepared by spiking before extraction.
[d] $(B/A) \times 100$.
[e] $(C/B) \times 100$.
[f] $(C/A) \times 100 = (\text{ME} \times \text{RE})/100$.

and *x* the concentration of the analyte. The mean standard deviation value for slope and intercept observed were 0.08 and 0.01 respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 99.1 to 101.4% and 1.2 to 4.4% respectively. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was found to be 0.20 μg/mL at a signal-to-noise ratio (S/N) of ≥97 with a limit of detection (LOD) of 0.0013 μg/mL.

The intra-batch and inter-batch precision and accuracy were established from validation runs performed at LLOQ QC, LQC, MQC-2, MQC-1, HQC and ULOQ QC levels (Table 1). The intra-batch precision (%CV) ranged from 1.1 to 6.8 and the accuracy was within 94.7 to 100.5%. For the inter-batch experiments, the precision varied from 3.6 to 7.8 and the accuracy was within 93.3 to 100.3%.

3.4. Recovery, matrix effect, matrix factor, ion suppression and reinjection reproducibility

The relative recovery, absolute matrix effect and process efficiency data for CYC at LQC, MQC-2, MQC-1 and HQC is presented in Table 2. The relative recovery of CYC is the 'true recovery', which is unaffected by the matrix as it is calculated by comparing the area ratio response (analyte/IS) of extracted (spiked before extraction) and unextracted (spiked after extraction) samples. The mean process efficiency/absolute recovery obtained for CYC was between 43.9% and 51.5% at all QC levels, while the recovery of IS was 97.4% with %CV of 3.9. Further, the more important parameter in the evaluation and validation of a bioanalytical method using biofluids is the demonstration of absence of 'relative' matrix effect, which compares the precision (%CV) values between different lots (sources) of plasma (spiked after extraction) samples. The precision (%CV) results varied from 0.6 to 7.4% for different plasma lots at LQC and HQC levels (Table 3). The average matrix factor value calculated as the response of post spiked sample/response of neat solutions in mobile phase at the LLOQ levels was 1.01, which indicates a minor suppression of 1%. Results of post-column infusion experiment in Fig. 3 indicate no ion suppression or enhancement at the retention time of CYC and IS. Also, the reproducibility of retention times for the analyte, expressed as %CV was ≤1% for 100 injections on the same column.

3.5. Stability and dilution integrity

Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions, simulating the same conditions which occurred during study sample analysis. The stock solution of CYC was stable at room temperature for 8 h and at 4 °C for 27 days. The intermedi-

Table 3
Relative matrix effect in different lots of human plasma at LQC and HQC levels for cycloserine ($n = 4$).

Plasma lots	LQC (0.60 μg/mL) Mean calculated conc.[a] (%CV)	HQC (22.50 μg/mL) Mean calculated conc.[a] (%CV)
Lot-1	0.54 (1.4)	21.42 (0.6)
Lot-2	0.58 (7.1)	20.51 (2.4)
Lot-3	0.56 (5.5)	21.35 (2.2)
Lot-4	0.55 (6.8)	20.23 (4.1)
Lot-5	0.55 (5.9)	21.09 (3.1)
Lot-6	0.55 (7.4)	20.07 (3.3)
Lot-7 (haemolysed)	0.55 (4.6)	20.50 (1.3)
Lot-8 (lipemic)	0.54 (4.1)	20.22 (3.5)

CV: coefficient of variation.
[a] Mean of four replicate observations at each concentration.

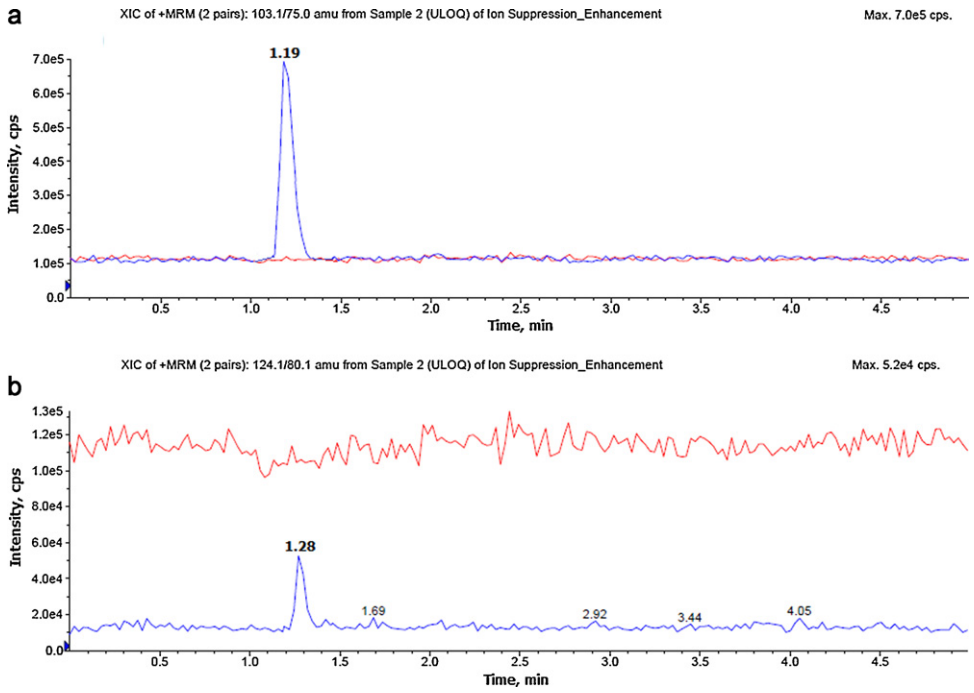

Fig. 3. Representative post column analyte infusion MRM LC–MS/MS overlaid chromatograms for cycloserine and niacin: (a) exact ion current (XIC) chromatogram of cycloserine (m/z 103.1 → 75.0) and (b) XIC of niacin (IS, m/z 124.1 → 80.1).

Table 4
Stability results for cycloserine under different conditions ($n = 6$).

Stability	Storage condition	Level	A^a (μg/mL)	%CV	% change[b]
Bench top stability	Room temperature (25 h)	LQC	0.62	1.7	3.3
		HQC	24.16	3.5	7.4
Processed sample stability (extracted samples)	Auto sampler (4 °C, 91 h)	LQC	0.62	2.9	3.3
		HQC	24.29	4.2	7.9
Processed sample stability (extracted samples)	Room temperature (91 h)	LQC	0.61	2.4	1.7
		HQC	24.03	0.8	6.8
Freeze and thaw stability	After 6th cycle at −20 °C	LQC	0.55	1.2	−8.3
		HQC	21.62	5.5	−3.9
Long term stability	91 days at −20 °C	LQC	0.58	7.3	−3.3
		HQC	21.24	7.9	−5.6

CV: coefficient of variance;
n: number of replicates at each level.
[a] Mean stability sample.
[b] XXX *%Change* = (*Mean stability samples–Mean comparison samples*/*Mean comparison samples*) × 100.

ate stock solutions of CYC in deionized water were stable at room temperature for 27 h and at 4 °C for 15 days with % change of 4.5% and 2.1% respectively. CYC was found stable in controlled plasma at room temperature up to 25 h and for six freeze and thaw cycles. The analyte in extracted plasma samples were stable for 91 h under refrigerated conditions (4 °C) and for 91 h under room temperature. The spiked plasma samples of CYC stored at −20 °C for long term stability were found stable for minimum period of 91 days. The values for the percent change for all the stability experiments are compiled in Table 4.

The precision values for dilution integrity of 1/10 of 5 × ULOQ (15.00 μg/mL) and 1/10 of HQC (2.25 μg/mL) concentration were 1.7 and 2.3%, while the accuracy results were within 99.2 and 102.6% respectively, which is within the acceptance limit of 15% for precision (%CV) and 85–115% for accuracy.

3.6. Application of the method on human subjects

The validated method was applied for a pharmacokinetic study of CYC in 24 healthy Indian adult male subjects who received 250 mg test and reference formulations of CYC under fasting condition. The method was sensitive enough to monitor their plasma concentration up to 48.0 h. In all approximately 1666 samples including the calibration, QC and volunteer samples were run and analyzed successfully. The precision and accuracy for calibration

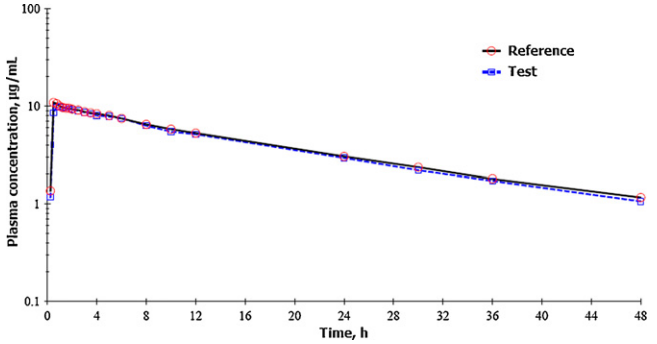

Fig. 4. Mean plasma concentration-time profile of cycloserine after oral administration of test (250 mg cycloserine capsule of an Indian Company) and a reference (SEROMYCIN®, 250 mg cycloserine capsule from Eli Lilly and Company) formulation to 24 healthy subjects under fasting condition.

Table 5
Mean pharmacokinetic parameters of cycloserine after oral administration of 250 mg cycloserine capsule formulation in 24 healthy Indian subjects under fasting condition.

Parameter	Test Mean ± SD	Reference Mean ± SD
C_{max} (μg/mL)	11.94 ± 2.00	12.56 ± 2.18
T_{max} (h)	1.06 ± 0.75	0.93 ± 0.65
$t_{1/2}$ (h)	15.14 ± 3.85	16.08 ± 3.71
$AUC_{0-48\,h}$ (h μg/mL)	176.73 ± 39.72	183.49 ± 40.01
AUC_{0-inf} (h μg/mL)	202.97 ± 54.43	213.14 ± 55.23
Kel (1/h)	0.05 ± 0.02	0.05 ± 0.02

C_{max}: maximum plasma concentration.
T_{max}: time point of maximum plasma concentration.
$t_{1/2}$: half life of drug elimination during the terminal phase.
AUC_{0-t}: area under the plasma concentration-time curve from zero hour to 48 h.
AUC_{0-inf}: area under the plasma concentration-time curve from zero hour to infinity.

Table 6
Incurred sample reanalysis data for cycloserine.

Sr. No.	Initial concentration (μg/mL)	Repeat concentration (μg/mL)	Mean	% difference
1	12.12	13.04	12.58	7.31
2	1.27	1.25	1.26	−1.67
3	0.68	0.66	0.67	−3.13
4	11.41	12.06	11.74	5.54
5	1.27	1.36	1.31	7.23
6	12.16	12.65	12.41	3.95
7	0.73	0.70	0.71	−4.57
8	12.32	12.8	12.56	3.82
9	1.17	1.11	1.14	−5.60
10	13.6	13.96	13.78	2.61
11	1.81	1.96	1.89	7.85
12	14.93	13.79	14.36	−7.94
13	15.08	14.15	14.62	−6.36
14	1.10	1.06	1.08	−3.53
15	15.59	16.23	15.91	4.02
16	1.02	1.06	1.04	4.23
17	11.63	11.56	11.60	−0.60
18	13.86	13.13	13.50	−5.41
19	1.16	1.08	1.12	−6.96
20	1.85	1.94	1.90	4.80

%Change = (Repeat value − Initial value/Mean of initial and repeat values) × 100.

and QC samples were well within the acceptable limits. The mean pharmacokinetic profile for the treatment, under fasting condition is presented in Fig. 4. A similar profile has been reported previously [17], for the same formulation given to 18 healthy subjects. However, the values of pharmacokinetic parameters were not computed in that report. The mean pharmacokinetic parameters obtained for the test and reference formulation in the present study are presented in Table 5. The % change in the randomly selected subject samples for incurred sample reanalysis was less than ±10% as shown in Table 6. This authenticates the reproducibility and ruggedness of the proposed method.

4. Conclusion

The objective of this work was to develop a selective, sensitive, rugged and a high throughput method for simultaneous estimation of CYC in human plasma, especially to meet the requirement for subject sample analysis. The solid phase extraction employed in the present work using Waters Oasis MCX cartridge gave consistent and reproducible recoveries for CYC. The run time per sample analysis of 5.0 min suggests high throughput of the proposed method. The maximum on-column loading at ULOQ was 60 ng for 2.0 μL injection volume. This was considerably less compared to other reported procedures [16,17], which helps in maintaining the efficiency and the lifetime of the column. Moreover, the limit of quantification is low enough to monitor at least five half-lives of CYC concentration with good intra and inter-assay reproducibility (%CV) for the quality controls. The sensitivity of the proposed method is adequate to support a wide range of pharmacokinetic/bioequivalence studies.

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