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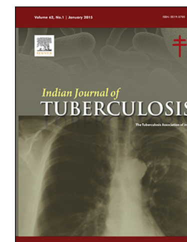

Original article

A selective and sensitive high performance liquid chromatography assay for the determination of cycloserine in human plasma

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ABSTRACT

Background: Cycloserine (CYC) is a second line antitubercular drug that is used for the treatment of multidrug resistant tuberculosis (MDR-TB) along with other antitubercular agents and is often used in developing countries. Monitoring CYC levels in plasma could be useful in the clinical management of patients with MDR-TB. A high performance liquid chromatography method for the determination of CYC in human plasma was developed.

Methods: The method involved extraction of the sample using solid phase extraction cartridges and analysis of the extracted sample using a reverse phase T3 column (150 mm) and detection at 240 nm with Photo Diode Array (PDA) detector. The chromatogram was run for 15 min at a flow rate of 0.4 ml/min at 30 °C.

Results and conclusion: The assay was specific for CYC and linear from 5.0 to 50.0 μg/ml. The relative standard deviations of within- and between-day assays were less than 10%. Recovery of CYC ranged from 102% to 109%. The interference of other second line anti-TB drugs in the assay of CYC was ruled out. The assay spans the concentration range of clinical interest. The specificity and sensitivity of this assay makes it highly suitable for pharmacokinetic studies.

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1. Introduction

Cycloserine (CYC) is an antibiotic produced by *Streptomyces garyphalus* and *Streptomyces orchidaceus* and is an analog of amino acid D-alanine. It inhibits enzymes D-alanine synthetase.[1,2] CYC is a second line antitubercular drug that is used for the treatment of multidrug resistant tuberculosis (MDR-TB) along with other antitubercular agents and is often used in developing countries.[3] After oral administration, CYC is readily absorbed from the gastro intestinal tract, with peak blood levels attained in 4–8 h.[4]

Resistance to second-line drugs is associated with worse treatment outcomes since an inadequate or poorly

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administered second line treatment regimen allows a drug-resistant strain to become dominant in a patient infected with MDR-TB. Therefore monitoring CYC levels in plasma could be useful in the clinical management of patients with MDR-TB.

Several analytical methods have been developed for the determination of CYC in plasma, which includes high performance liquid chromatographic (HPLC) methods and liquid chromatographic mass spectrometric (LCMS/MS) methods. The HPLC methods reported were based on derivatisation technique and requires special columns and large volume of samples.[3,5–7] Several LCMS/MS methods for the quantification of CYC in plasma have been reported.[8–10] However, these expensive techniques are not affordable in developing countries and for resource poor settings. Moreover none of these methods have included second-line anti-TB and anti-retroviral drugs in their specificity experiments. Since CYC is used in combination with these drugs in TB patients with and without HIV, it is essential to rule out the interference of these drugs in the development of method for the determination of CYC in plasma. We developed and validated a simple, selective and sensitive HPLC method for the determination of CYC.

2. Materials and methods

2.1. Chemicals

Pure CYC powder was obtained from Sigma–Aldrich Chemical Company, St. Louis, MO, USA. MCX (1 cc/30 mg) cartridges from Waters India, acetonitrile (HPLC grade), isopropyl alcohol (IPA), formic acid, sodium dihydrogen orthophosphate (NaH2PO4) and disodium hydrogen orthophosphate were purchased from Qualigens (India). Ammonia solution was obtained from SD Fine Chemicals Limited. Deionized water was processed through a water purification system (Siemens, Germany). Pooled human plasma was obtained from a Blood Bank, Chennai, India.

2.2. Chromatographic system

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), Photo diode array detector (SPD-M10Avp) and auto sampler (SIL-HTA) with built in system controller. Class VP-LC workstation was used for data collection and acquisition. The analytical column used was Atlantis T3, 150 mm × 4.6 mm ID, 3 μm particle size (Waters, Ireland) protected by a compatible guard column.

An isocratic mobile phase consisted of a mixture of 10 mM phosphate buffer (sodium dihydrogen phosphate NaH_2PO_4 – 3.19 g and disodium hydrogen orthophosphate, Na_2HPO_4 – 10.99 g in 1000 ml water) and acetonitrile:IPA (90:10) in the ratio of 95:5 (v/v), was used to separate the analyte from the endogenous components. Prior to preparation of the mobile phase, the solvents were degassed separately using a Millipore vacuum pump. The PDA detector was set at a wavelength of 240 nm. The chromatogram was run for 15 min at a flow rate of 0.4 ml/min at 30 °C. Unknown concentrations were derived from linear regression analysis vs. concentration curve. The linearity was verified using estimates of correlation coefficient (*r*).

2.3. Preparation of standard solution

A stock standard (1 mg/ml) was prepared by dissolving CYC in water. The working standards of CYC in concentrations ranging from 5.0 to 50.0 μg/ml were prepared in pooled plasma.

2.4. Sample preparation

To 200 μl each of calibration standards and test samples, 200 μl of 1% formic acid was added and the contents were vortexed vigorously, centrifuged at 10,000 rpm for 10 min. The analyte was extracted through solid phase extraction cartridges. The eluted solution was evaporated to dryness. The dried residue was reconstituted in 200 μl of diluent (90% water:10% of acetonitrile:IPA; 90:10) and 50 μl was injected into the HPLC column.

2.5. Method validation

2.5.1. Accuracy and linearity

The accuracy and linearity of CYC standards were evaluated by analysing a set of standards ranging from 5.0 to 50.0 μg/ml. The within day and between day variations were determined by processing each standard concentration in duplicate for six consecutive days.

2.5.2. Precision

In order to evaluate the precision of the method, plasma samples containing varying concentrations of CYC were analyzed in duplicate on three consecutive days.

2.5.3. Recovery

Known concentrations of CYC (2.5, 7.5 and 10.0 μg/ml) were prepared in pooled human plasma and were spiked with lower and higher concentrations of standards. The percentage of drug recovery from plasma samples was calculated by dividing the difference in CYC concentrations by the added concentration. Recovery experiments were carried out on three different occasions.

2.5.4. Specificity

Interference from endogenous compounds was investigated by analysing blank plasma samples. Interference from certain anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, ethionamide, levofloxacin and certain antiretroviral drugs, namely, nevirapine, efavirenz, zidovudine, didanosine, stavudine, lamivudine, saquinavir, lopinavir, ritonavir and indinavir at a concentration of 10 μg/ml was also evaluated.

2.5.5. Limits of detection (LOD) and quantitation (LOQ)

These values were estimated mathematically from the standard curve equations.[11] LOD was calculated using the formula $3.3 \times \sigma/S$, where σ is the standard deviation of Y-axis intercepts and S is the slope of the calibration curve. LOQ was calculated using the formula $10.0 \times \sigma/S$, where σ is the standard deviation of Y-axis intercepts and S is the slope of the calibration curve.

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