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Sensitive Ultra-performance Liquid Chromatography Tandem Mass Spectrometry Method for Determination of Cycloserine in Plasma for a Pharmacokinetics Study

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Abstract

A simple and sensitive ultra-performance liquid chromatography tandem mass spectrometry method has been developed and validated for the analysis of cycloserine in patients' plasma. Using methanol, cyloserine and propranolol (internal standard (IS)) was extracted from plasma by protein precipitation procedure. The chromatographic separation was successfully achieved on Phenomenex Kinetex^{TM} PFP C_{18} (2.1 mm × 100 mm, 2.6 μm) reversed-phase column. Acidified with 0.1% formic acid, water and acetonitrile were used as mobile phases for gradient elution. Cycloserine and IS were detected by Xevo® TQ MS triple quadrupole tandem mass spectrometer. The transition of protonated precursor to product ion were monitored at 103 → 75 *m/z* and 260.2 → 183 *m/z* for cycloserine and IS, respectively. The lower limit of quantification was 0.01 μg/mL. The method was linear over the concentration range 0.01–50 μg/mL with average coefficient of determination of 0.9994. The within-run and between-run precision and accuracy were in the range 3.7–19.3% (RSD) and 98.7–117.3%, respectively. Processed cycloserine sample was stable for 48 hours at 8°C and after three freeze–thaw cycles. The extraction efficiency ranged between 88.7 and 91.2%. The method was successfully applied in a pharmacokinetic study for the determination of cycloserine in plasma of patients with drug-resistant tuberculosis.

Introduction

Cycloserine is a structural analog of D-alanine, a broad-spectrum antibiotic, naturally produced from *Streptomyces lavendulae* and *Streptomyces garyphalus* (1). It is clinically used as the second-line drug for treatment of multidrug-resistant tuberculosis (2). Cycloserine exerts bacteriostatic (3) action by preventing the biosynthesis of peptidoglycan through inhibition of D-alanine:D-alanine ligase and alanine racemase of the *Mycobacterium tuberculosis* (4).

Cycloserine is water soluble and absorbed faster in healthy individuals than tuberculosis patients (5, 6). It distributes widely in body fluids and tissues. Primarily the kidneys excrete it mostly in unchanged form. The clearance is lower in healthy individuals than in tuberculosis patients (7). Although cycloserine is effective in treating resistant strains of *Mycobacterium tuberculosis*, its use is limited due to severe toxicities on the kidney and neuropsychiatric adverse reactions (2, 3). Consequently, the World Health Organization (2) recommends monitoring of cycloserine plasma concentration to ensure that the peak concentration is kept below 35 μg/mL.

Determination of plasma concentration of cycloserine or any drug requires a method that has less steps in sample preparation and better sensitivity. Several bioanalytical methods for cycloserine employing LC–MS-MS have been described in literature (8–11) with sensitivities in the range 0.05–0.5 μg/mL. The other HPLC–MS and HPLC–MS-MS methods had sensitivities of 0.5 and 0.2 μg/mL, respectively (12, 13). Three of these methods employed solid phase extraction procedure, which is expensive and time consuming. The other methods made use of protein precipitation and derivatisation (8, 10, 12) that involved several steps and used very small volumes

of reagents. More errors are likely to be made if small volumes of reagents are used in sample processing, which eventually affect precision.

The only UPLC–MS-MS method (14) for cycloserine analysis available has two shortcomings. Firstly, many steps are involved when mixing volumes of reagents. Moreover, the method required a sample to be centrifuged twice before it is ready for analysis. Secondly, as small as 4 and 5 µL volumes of reagents were used in sample preparation process. These pitfalls compromise the precision and accuracy especially if the method is adapted for routine batch analysis of clinical samples. The objective of this study was to develop a bioanalytical method that has less steps in sample preparation, simple as well as better sensitivity for analysis of cycloserine in plasma of patients.

Therefore, in the current study a simple, stable and sensitive UPLC–MS-MS method was developed and validated for the determination of cycloserine in plasma. The method was applied to quantify cycloserine as a metabolite of terizidone in pharmacokinetic study of patients with drug-resistant tuberculosis.

Experimental

Chemical reagents

The reference standard, cycloserine (Figure 1) powder, analytical grade (CAS: 68-41-7) and propranolol powder (internal standard (IS), Figure 1) analytical grade (Lot: 29H4016) were purchased from Sigma-Aldrich, Germany. The HPLC grade of methanol and acetonitrile were purchased from Merck (South Africa) and Sigma-Aldrich (Germany), respectively. Formic acid was acquired from Merck, South Africa. Water (18 mΩ) was obtained from a Direct-Q3 (Mmillipore) water purification system. Blank pooled plasma (for research purpose) was purchased from Sigma-Aldrich, Germany.

Instrumentation

The analytical equipment consisted of a Waters® Acquity (Waters, Milford, MA, USA) ultra-performance liquid chromatography (UPLC) system. It consisted of a binary solvent manager with two independent pump systems that pumped solvent through the system with a maximum pressure of 15,000 psi. The Sample manager injected samples that were drawn from the vials located in the Sample organizer onto the chromatographic column. The system was also equipped with a column heater. Temperature in the Sample organizer was kept at 10°C. Coupled to the UPLC system was a Xevo® TQ MS triple quadrupole tandem mass spectrometer (atmospheric pressure ionization) and was used for high-resolution UPLC–MS-MS analysis. The MassLynx™ software, acquired, managed and processed the mass spectrometry data and UPLC instrument control.

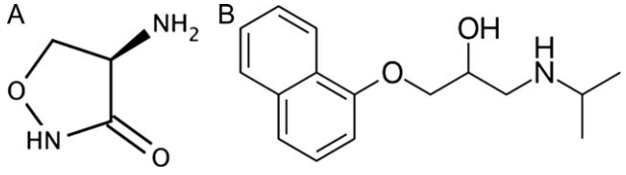

Figure 1. Chemical structure of cycloserine (A) and propranolol (B).

Chromatographic and mass spectrometry conditions

Separation of cycloserine and propranolol was carried out on Phenomenex Kinetex™ PFP C_{18} (2.1 mm × 100 mm, 2.6 µm) reversed-phase column. The column temperature was maintained at 45°C. The mobile phases consisted of water (A) and acetonitrile (B) both acidified with 0.1% formic acid. The gradient steps in Table I were used to elute cycloserine and propranolol at flow rate of 0.4 mL/min. The total chromatographic run time was 7 min.

Ionisation, detection and quantification of cycloserine and IS was achieved on Xevo® TQ MS triple quadrupole tandem mass spectrometer equipped with electrospray ionization source operating in positive ion mode. The mass spectrometer performed the detection of cycloserine and IS using multiple reaction mode (MRM) with the transition (precursor → product) of 103 → 75 *m/z* for cycloserine and 260.2 → 183 *m/z* for IS. The operating parameters used were set as follows: capillary voltage, 3.5 V; cone voltage, 15 V; collision energy range, 7–15 eV; source temperature, 140°C; desolvation temperature, 400°C; desolvation gas, 800 L/h and cone gas, 50 L/h.

Preparation of stock solutions, quality controls and calibration standards

Stock solution of cycloserine (1,000 µg/mL) was prepared by dissolving accurately weighed 10.0 mg cycloserine in 10 mL of methanol. The stock quality control (QC) cycloserine solution with concentration of 800 µg/mL was prepared by dissolving 8.0 mg of cycloserine in 10 mL of methanol. The IS stock solution with a concentration of 100 µg/mL was prepared by dissolving 10 mg of propranolol in 100 mL of methanol. These solutions were kept at −80°C.

The stock solution of cycloserine was appropriately diluted with methanol in order to make eight concentrations of standard working solution (500, 250, 50, 10, 2, 1, 0.5, 0.1 µg/mL). Calibration standards were prepared by spiking 100 µL of each standard working solution to 900 µL of blank plasma. In this way, eight concentrations (50, 25, 5, 1, 0.2, 0.1, 0.05, 0.01 µg/mL) for the calibration standard curve were prepared. The QC working standard solution was prepared by appropriate dilution of stock QC solution with methanol to 400, 100 and 5 µg/mL. The QC samples were prepared at concentrations of 40, 10 and 0.5 µg/mL for high QC, medium QC and low QC, respectively, by spiking 100 µL of each QC working solution with 900 µL of blank plasma. The IS solution was further diluted with methanol to the concentration of 0.01 µg/mL.

Plasma sample pre-treatment

Extraction of cycloserine from the spiked plasma (calibration standard curve and QC samples) was achieved through plasma protein precipitation. To each 200 µL of spiked plasma, 800 µL of IS solution was added and vortex mixed for 1 min. In order to separate the precipitate, the contents were centrifuged (13,000 rpm) for 10 min

Table I. Gradient Elution Steps

Time (min)	%Water (A)	%Acetonitrile (B)
0	100	0
0.5	100	0
4.5	90	10
6	0	100
6.5	0	100
6.51	100	0
7	100	0

at 2°C. The 500 µL of the supernatant were pipetted into a clean vial. A further 500 µL of water was added to the supernatant and vortex mixed. Finally, 2 µL of this solution was injected onto the UPLC system.

Method validation

The bioanalytical method was validated according to the International Conference for Harmonisation and United States Foods and Drugs Administration guidelines for validation of analytical procedures (15, 16). The method was validated for sensitivity or limit of quantification, limit of detection, linearity, accuracy, precision, recovery, carry-over, matrix effect and stability.

Analysis of patient's plasma samples

Plasma cycloserine (terizidone metabolite) concentration was determined in 78 drug-resistant tuberculosis patients treated with 750 mg terizidone daily dose and other anti-tuberculosis drugs. The study was approved by the ethics committees of University of Cape Town (Ref: 777/2014) and University of the Western Cape (Ref: 07/6/12). Frozen patients' plasma samples were left to thaw at room temperature and vortex mixed before pipetting. To every 200 µL of plasma, 800 µL of IS were added and vortex mixed for 1 min. The procedure for the rest of the patients' plasma sample preparation was same as the calibration standards. The bioanalytical run consisted of the processed sample plasma without IS and terizidone, processed plasma with IS only, calibration standards, processed patient samples and QC samples.

Results

Chromatographic and mass spectrometry conditions optimization

In order to optimize the separation efficiency and chromatographic peak shape of the analyte and the IS, several mobile phase compositions, the flow rate and different C_{18} columns were employed. Acetonitrile and water, both acidified with 0.1% formic acid were found to be the best mobile phases with gradient composition shown in Table I. Additionally, the flow rate of 0.4 mL/min was found suitable for current method. The column, Acquity UPLC BEH C_{18} (2.1 mm × 100 mm, 1.7 µm), was tried but the analyte eluted quite early. Separation of cycloserine and IS was best achieved on a Phenomenex Kinetex™ PFP C_{18} (2.1 mm × 100 mm, 2.6 µm) reversed-phase column as it is ideal for separation of polar compounds.

Using electrospray ionization in positive mode, the protonated precursor ions $[M+H]^+$ of cycloserine and propranolol that were dominant had *m/z* ratios of 103 and 260.2, respectively. After the conditions for fragmentation were optimized with collision energy in the range 7–15 eV, the most abundant and stable ions in the product spectra were observed at *m/z* ratios of 75 and 183 for cycloserine and propranolol, respectively. Other parameters for ionization were optimized to obtain highest, stable and consistent signal intensity for cycloserine and propranolol.

Method validation

Sensitivity and limit of detection

Sensitivity was the lowest cycloserine concentration (lower limit of quantification—LLOQ) that was measured with acceptable precision and accuracy. The lowest concentration of the calibration standards was determined to be 0.01 µg/mL as it had a precision of 19.3% (percentage relative standard deviation—%RSD) and accuracy of 98.7% (Table III). This was within the acceptable precision of not more than 20% and accuracy within 80–120% (15).

The limit of detection (LOD) based on the calibration curve, was calculated using the following formula:

$$\mathrm{LOD} = 3.3\sigma/S \quad (1)$$

where σ is the standard deviation of the *Y*-intercepts of the regression equations shown in Table II. Similarly, *S* is the average of the slopes of the regression equations. The value of the LOD was 0.004 µg/mL.

Linearity

The 8-point standard calibration curve was constructed by ploting cycloserine nomimal concentrations on the *X*-axis against peak area ratios of cycloserine and IS on the *Y*-axis (response). The weighting factor of $1/x$ improved the linear regression fit. Linearity was assessed statistically by fitting the data (nominal concentration and response) to a linear regression model by method of least squares. The regression equations constructed from six replicate bioanalytical runs of the eight calibration standards are shown in Table II together with the corresponding coefficient of determination values and the sum of residuals. The average coefficient of determination and correlation coefficient was 0.9994 and 0.9997, respectively.

Accuracy and precision

Five replicate values from the analyses of LLOQ, low QC, median QC and high QC were employed to calculate accuracy of the assay.

Table II. Bioanalytical Method Linearity Parameters

Run number	*R*-square (r^2)	Linear equation	Residual sum of squares
1	0.9998	$Y = 4.202x + 0.0604$	0.00069
2	0.9998	$Y = 4.161x + 0.0511$	0.00107
3	0.9999	$Y = 4.244x + 0.0536$	0.00093
4	0.9996	$Y = 4.301x + 0.0489$	0.00141
5	0.9998	$Y = 4.215x + 0.0578$	0.00022
6	0.9989	$Y = 4.207x + 0.0621$	0.00209

Table III. Within-Run and Between-Run Accuracy and Precision of the LLOQ, Low QC, Median QC and High QC Assay

Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	Accuracy (%)	Precision (%RSD)
Within-run analysis			
0.01	0.0098 ± 0.002	98.7	19.3
0.5	0.58 ± 0.062	114.6	10.8
10	9.94 ± 0.697	99.4	7
40	40.48 ± 1.48	101.2	3.7
Between-run analysis			
0.01	0.012 ± 0.002	117.3	15
0.5	0.57 ± 0.03	111	5.2
10	10.3 ± 0.674	103	6.5
40	39.6 ± 1.06	99	2.6

SD, standard deviation.

The accuracy was estimated as the percentage ratio average (five replicates) of the back-calculated concentrations and the nominal concentration. The accuracy ranged from 98.7–116% to 99–117% for within-run analysis and between-run analysis, respectively (Table III). Precision (%RSD) was calculated as the percentage ratio of the standard deviation and the mean of the replicates analyzed on same day (within-run) and different days (between-run). The within-run precision ranged between 3.7 and 19.3% while between-run precision was in the range 2.6–15% (Table III). The accuracy and precision was within the accepted range (15).

Carry-over and matrix effect

The carry-over was assessed by injecting the highest concentration of the calibration standard (50 µg/mL) followed by a blank sample. A response of 238.874 was read after injection of 50 µg/mL and 0.00677 after injection of blank sample. The blank sample response corresponded to cycloserine concentration of 0.0021 µg/mL and was below the LLOQ.

The matrix effect was evaluated by comparing the instrument response (peak area) of cycloserine and IS that was prepared in methanol and the one spiked in plasma. The average ratios of the peak area of cycloserine spiked in plasma to the one prepared in methanol were 0.9927 and 1.003 for low QC and high QC, respectively. Similarly, the average IS ratios were 0.9961 and 1.01. These results show that the effect of plasma (matrix) on the ionization of both cycloserine and IS was not significant.

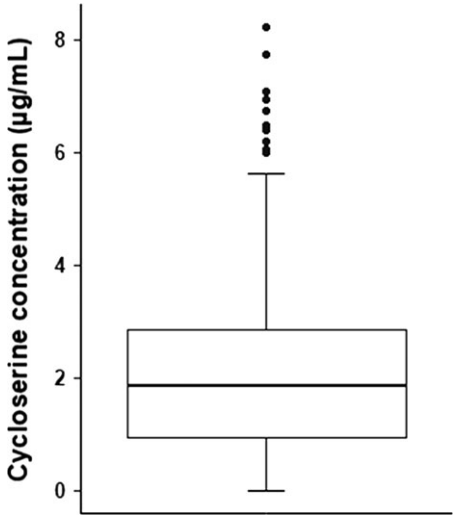

Figure 2. Distribution of cycloserine concentrations in drug-resistant tuberculosis patients.

Stability and recovery

The analyte stability was evaluated according to the situations that were expected to be experienced during patient plasma sample processing and analysis (15). These situations were freeze–thaw of at least one cycle and stability of processed samples in the autosampler for maximum of 24 h. The mean cycloserine concentration for the low QC and high QC after three freeze–thaw cycles was almost close to the nominal concentration. Nevertheless, the mean concentration of the processed samples that were stored at 2–8°C dropped by 11.9 and 1.75% for low QC and high QC, respectively, after 48 h. The mean accuracy was 88.1 and 98.3% while precision was 9.1 and 3.7% (RSD) the low QC and high QC, respectively. Recovery was determined by comparing instrument response of the analyte that was prepared in the solvent with analyte extracted from plasma (15). The percentage recovery was in the range 88.7–91.2 for both low and high QC.

Application of bioanalytical method in pharmacokinetic study

A total of 78 patients participated in the study after signing informed consent form. They provided 608 plasma samples, which were analyzed for cycloserine as a metabolite of terizidone. The mean and median concentrations were 2.1 ± 1.5 µg/mL and 1.9 (0.01–8.2) µg/mL, respectively. The distribution of the cycloserine concentration is shown in Figure 2.

Discussion

Protein precipitation was the preferred method of analyte (cycloserine) extraction from plasma as it is simple, fast and inexpensive (17). The 2-fold dilution of the supernatant with water and 5-fold in protein precipitation procedure ensured also the dilution of interfering compounds. This procedure reduced the matrix effect (10).

In the chromatographic separation, the initial 100% aqueous phase favored the elution of cycloserine first as it is more polar than the IS. Subsequently, the gradual reduction of the aqueous phase or gradual increase of the organic phase in the second step of the

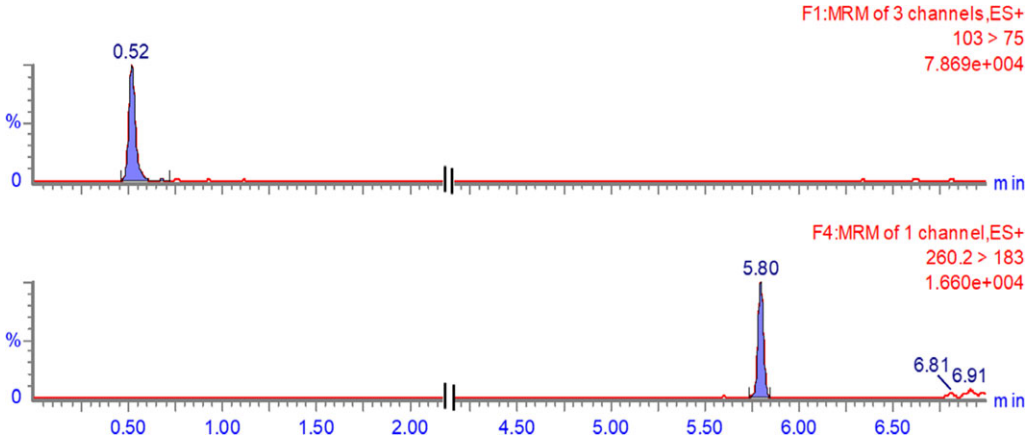

Figure 3. Chromatograms of cycloserine and propranolol with retention times at 0.52 and 5.8 min, respectively.

gradient favored the elution of the IS. The excellent peak shape, better separation and reproducibility (Figure 3) resulted from the combined C_{18} and pentafluoropheny (PFP) functionality through pi–pi, hydrogen bonding, dipole–dipole and hydrophobic interactions. Hence, the PFP C_{18} (2.1 mm × 100 mm, 2.6 µm) reversed-phase column was the best of the columns that were tried.

The carry-over effect (0.0021 µg/mL), although minimal, was rather expected because cycloserine molecule is polar and has affinity for pentafluoropheny moiety (stationary phase component). Hence, it remained stuck in the column but was gradually eluting with each run. On the other hand, the calibration curve was linear (16) over the concentration range 0.01–50 µg/mL as the coefficient of determination value and the sum of the residuals was approximately one and zero, respectively.

Furthermore, our method had a better sensitivity of 0.01 µg/mL than a previously reported UPLC–MS-MS method (14) with 0.5 µg/mL. Hence, it was suitable to measure low concentrations of cycloserine as a metabolite of terizidone in patients who were slow metabolisers. The method was successfully used to determine plasma concentrations of cycloserine in a population pharmacokinetic study of patients with drug-resistant tuberculosis hospitalized for intensive phase of treatment.

Conclusion

The simple, sensitive and reliable UPLC–MS-MS method was developed and validated for analysis of cycloserine in plasma of patients with drug-resistant tuberculosis. The validation parameters indicated that the method was linear, sensitive, stable, precise and accurate. The method is suitable for pharmacokinetic studies as sample processing is relatively simple and does not require use of expensive chemicals.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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