Original article

An LC-MS/MS Method for the Determination of Cycloserine in Human Plasma : Its Application to a Pharmacokinetic Study

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Abstract

A simple and rapid liquid chromatography-tandem mass spectrometric (LC-MS/MS) method was developed and validated for the determination of cycloserine in human plasma. Carbamazepine was used as an internal standard. The method utilized protein precipitation with acetonitrile followed by dilution with 50% methanol. The separation was performed on a C18 column using methanol-0.1% formic acid (70:30, v/v) as the mobile phase. Detection was performed on a quadrupole mass spectrometer using an electrospray ionization interface in multiple reaction monitoring mode. The method was proven to be specific, sensitive, accurate, precise, and linear over the concentration range of 0.3-70 µg/mL with correlation coefficients greater than 0.995. The intra- and interday precisions and accuracies were within 10.7%. The method was successfully applied to a pharmacokinetic study of cycloserine following the oral administration of 250 and 750 mg cycloserine capsules in 11 healthy Thai volunteers under fasting conditions.

Keywords: Cycloserine, human plasma, LC-MS/MS, pharmacokinetic

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Introduction

Cycloserine, a structural analog of D-alanine amino acid, was recommended by the World Health Organization guidelines as one of the core second-line agents, and it is used in conjunction with other anti-tubercular agents in the treatment of multidrug resistance tuberculosis (WHO, 2016). Cycloserine targets sequential bacterial cell wall peptidoglycan biosynthesis enzymes (Batson *et al*., 2017). When administered orally, cycloserine is rapidly absorbed at a Tmax of 0.75 hours in the fasting state (Kim *et al*., 2000; Zhu, Nix, Adam, Childs, & Peloquin, 2001). It is widely distributed in the body fluids and tissues and excreted by the kidney in unchanged form (Brennan, Young, & Robertson, 2008). The terminal half-life of cycloserine is 8 to 12 hours (Kim *et al*., 2000).

Several analytical methods have been reported for the determination of cycloserine in human plasma, and in biological fluids. These methods are based on spectrophotometric (David, Ionescu, & Dumitrescu, 2001; Kumar, Polisetty, Sudha, Vijayakumar, & Ramachandran, 2018), voltammetric (Pattar and Nandibewoor, 2016) and chromatographic (Patel *et al*., 2011; Han *et al*., 2013; Polagani, Pilli, Maddela, Gajula, & Gandu, 2013; Yaroshenko, Grigoriev, & Sidorova, 2014; Park *et al*, 2015; Zhou *et al*., 2015; Stepanova, Ovcharov, Barsegyan, & Chistyakov, 2016; Mao *et al*., 2017; Zhang *et al*., 2018; Mulubwa & Mugabo, 2019) approaches. Liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods have advantages over other methods in terms of the simpler and faster preparation procedure, shorter analytical times and higher selectivity. The purpose of this study was to develop and validate an LC-MS/MS method for the determination of cycloserine in human plasma and apply it to a pharmacokinetic study of cycloserine following the oral administration of 250 and 750 mg cycloserine capsules in 11 healthy Thai volunteers under fasting conditions.

Materials and Methods

Chemicals

Cycloserine and carbamazepine were purchased from the United States Pharmacopeial Convention (Rockville, MD, USA). HPLC-grade methanol and LC-MS grade formic acid were obtained from Honeywell Specialty Chemicals Seelze GmbH, Germany. Ultrapure water generated by Milli-Q (Millipore Corporation, Bedford, MA, USA) was used. All other chemicals used in this study were of analytical grade. Drug-free human plasma was obtained from the Clinical Research Unit, Department of Medical Sciences (Nonthaburi, Thailand).

Instrumentation and chromatographic conditions

The chromatographic separation was performed on an Acclaim™ 120 C18 column (3 µm, 100 × 2.1 mm i.d., Dionex, Sunnyvale, CA, USA) protected by a C18 guard column (5 × 2.1 mm i.d., Waters Corporation, Milford, MA, USA). The mobile phase was a mixture of 0.1% formic acid and methanol (30:70, v/v) at a flow rate of 0.15 mL/min. The column and autosampler were set at 40ºC and 10ºC, respectively.

A Waters Xevo TQD Tandem Mass Spectrometer (Waters Corporation, Milford, MA, USA) was operated with an electrospray ionization source in positive ionization mode. Quantification was performed with the multiple reaction monitoring (MRM) mode of the transitions of *m/z* 103.02□74.98 for cycloserine and *m/z* 237.01□194.02 for carbamazepine. The cone voltage and collision energy (CE) were optimized as 20 V

and 6 eV, respectively, for cycloserine and 38 V and 18 eV, respectively, for carbamazepine. The other MS parameters were as follows: capillary voltage 3.7 kV; desolvation temperature 250°C; source temperature 150°C; ion spray voltage 4,000 V; and desolvation gas flow 900 L/h. Argon was used as the collision gas. Integration of the peak area and data analysis were performed using MassLynx V4.1SCN918.

Preparation of calibration curves and quality control samples

Stock solutions (1000 µg/mL) of cycloserine and an internal standard (IS), carbamazepine, were separately prepared in methanol. Working solutions of cycloserine and IS were prepared by diluting stock solutions with 50% methanol. Calibration standards were prepared by spiking 90 µL blank plasma with 10 µL cycloserine working solutions to produce the final concentrations of 0.3, 0.6, 0.9, 3.0, 6.0, 9.0, 30.0, 60.0 and 70.0 µg/mL. QC samples were prepared at the lower limit of quantification (LLOQ), low (QCL), medium (QCM), and high (QCH) concentrations of 0.3, 0.6, 6.0 and 60.0 µg/mL, respectively.

Sample preparation

All frozen human plasma samples obtained from a pharmacokinetic study of cycloserine following the oral administration of 250 and 750 mg cycloserine capsules in 11 healthy Thai volunteers under fasting conditions were thawed at room temperature and vortexed mixed. An aliquot of 100 µL of plasma was pipetted into a 1.5 mL microcentrifuge tube. Twenty microliters of IS solution (10.0 µg/mL) was added and vortex mixed. Then, 700 µL of methanol was added and vortex mixed, followed by centrifugation at 4ºC, 12,000 rpm for 10 min. An aliquot of 100 µL of the supernatant was diluted with 400 µL of 50% methanol and vortex mixed. Finally, an aliquot of 1 µL was injected into the LC-MS/MS system.

Method validation

The method was validated according to the U.S. Food and Drug Administration bioanalytical method validation guidance (2018) on the selectivity, carry over effect, linearity, precision, accuracy, recovery, dilution integrity, matrix effect and stability. The selectivity was evaluated using six different batches of blank plasma. Any endogenous peak found in blank plasma at the retention time of the analyte should be less than 20% of the mean response of the analyte in the extracted LLOQ whereas that at the retention time of the IS should be less than 5% of the mean response of the IS. Carryover was assessed by injecting blank samples after the injection of an upper limit of quantification (ULOQ) sample. Carry over in the blank sample following the high concentration standard should not be greater than 20% of the LLOQ and 5% for the IS. The calibration curves were generated by plotting drug to IS peak area ratios against drug concentrations using weighted (1/concentration) least-squares linear regression. The intraday accuracy and precision were determined using six replicates of QCs at four concentration levels (LLOQ, QCL, QCM, and QCH). The interday accuracy and precision were determined for three days. Accuracy expressed as the relative error (RE) and precision expressed as the coefficient of variation (CV) should be within 15%, except at the LLOQ where it should be less than 20%. Recovery was evaluated using six replicates at QCL, QCM, and QCH by comparing the mean peak areas of the extracted QCs with those of the extracted blank plasma postfortified with QC working solutions. The dilution integrity was evaluated by preparing QC samples at a concentration approximately 1.7-times the

highest concentration of the calibration curve (ULOQ). These samples were further diluted by 2-fold and 4-fold with blank plasma and assayed along with calibration standards. The accuracy and precision of the dilution integrity samples should be within 15%. The matrix effect was assessed using six replicates of QCL and QCH in six different batches of blank plasma, including normal, lipemic, and hemolyzed plasma. Accuracy and precision within 15% indicated "no matrix effect". The stability of cycloserine in plasma was evaluated for short-term and long-term storage, after three freeze-thaw cycles and postpreparative stability. The stability of cycloserine and IS stock solutions was also determined.

Method application

The proposed method was applied to determine the cycloserine concentrations in the plasma samples of 11 healthy Thai volunteers after the oral administration of cycloserine capsules (250 and 750 mg) under fasting conditions. The study protocol was approved by the Ethics Committees of the Institute for the Development of Human Research Protections, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. Blood samples (4 mL) were collected in K_2 EDTA tubes at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 24, 48 and 72 h after dosing and centrifuged at 3,000 x g and 4°C, for 10 min. Plasma samples were separated and kept frozen at -80°C until analysis. The plasma concentration-time profiles obtained from the subjects were analyzed by noncompartmental analysis using Phoenix WinNonlin 6.3 software.

Results

Selectivity

The mass spectra of cycloserine and IS are presented in Figure 1. Under the described chromatographic conditions, the retention times of cycloserine and IS were approximately 1.4 and 2.7 min, respectively. No peak interferences were observed in six different sources of human plasma, as well as in all study samples. Figure 2 shows the chromatograms obtained from blank plasma, spiked plasma at the LLOQ and a plasma sample from a volunteer obtained at 1 h after the oral administration of a single dose of 750 mg cycloserine capsules, indicating the selectivity of the method for routine sample analysis.

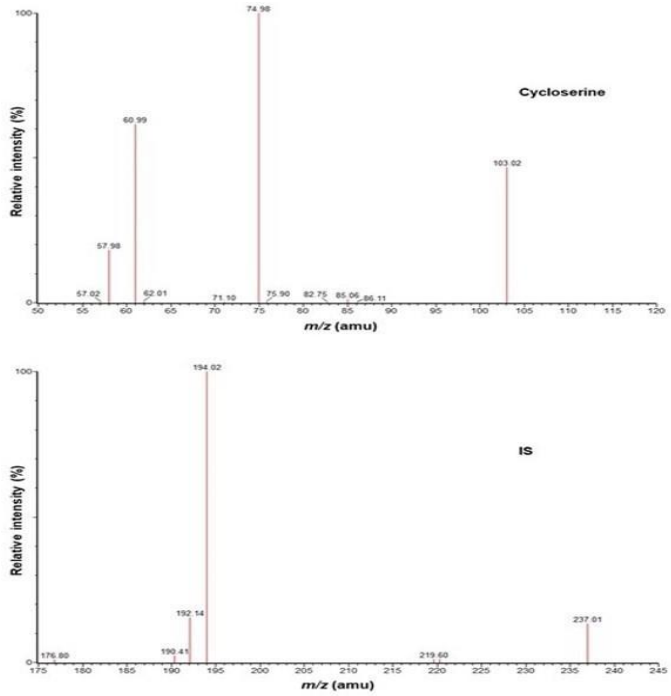

Figure 1. Mass spectra of cycloserine and carbamazepine (IS)

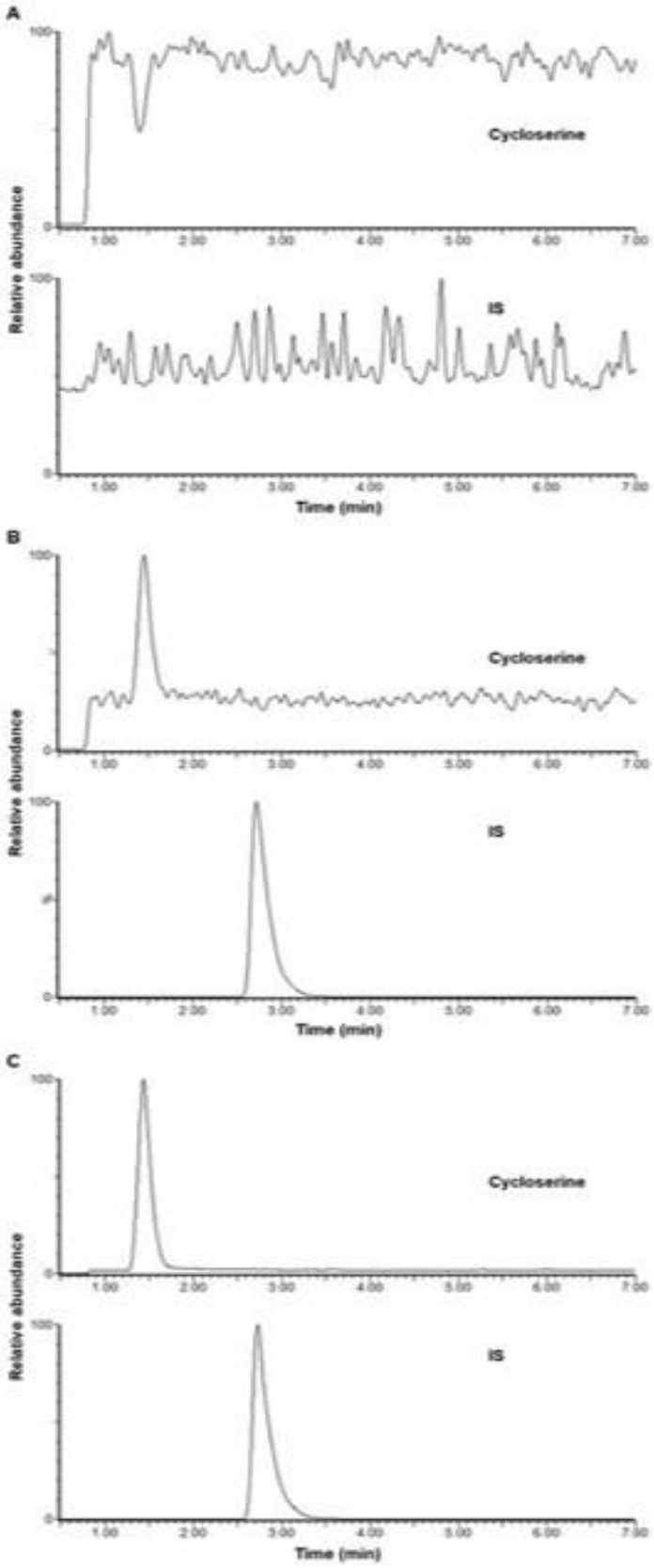

Figure 2. Typical MRM chromatograms of blank human plasma (A); blank human plasma spiked with cycloserine at LLOQ (0.3 µg/mL) and carbamazepine as IS (B); and plasma sample of a volunteer at 1 h after oral administration of 750 mg cycloserine (C).

Carry over effect

No peak interferences were observed in the blank samples following the injection of calibration sample at ULOQ, indicating no carry over effect observed in the present method.

Linearity

Linear regression was performed with a weighting factor of 1/concentration. The method exhibited good linearity over a concentration range of 0.3-70 µg/mL with correlation coefficients greater than 0.999 as determined on three different days. The back-calculated concentrations of calibration standards were within the acceptance range of 15% of nominal values, including the LLOQ.

Accuracy and precision

As shown in Table 1, the intraday accuracy ranged from -3.2 to 10.7%, whereas the precision was less than 10.7%. The interday accuracy ranged from 2.8 to 5.4%, and the precision was less than 8.3%. These results indicated that the method is accurate and reproducible.

Table 1. Intra and interday accuracy and precision for cycloserine determination in human plasma.

		Cycloserine concentration (µg/mL)			
		0.3	**0.6**	**6.0**	**60.0**
Intraday (n=6)					
Day 1	Mean ± SD	0.3 ± 0.0	0.7 ± 0.0	6.6 ± 0.1	63.4 ± 2.2
	RE (%)	10.5	10.7	9.9	5.6
	CV (%)	2.6	2.4	1.3	3.5
Day 2	Mean ± SD	0.3 ± 0.0	0.6 ± 0.0	5.9 ± 0.1	61.7 ± 1.4
	RE (%)	5.7	-3.2	-0.9	2.8
	CV (%)	8.0	7.7	0.9	2.3
Day 3	Mean ± SD	0.3 ± 0.0	0.6 ± 0.0	6.3 ± 0.3	59.9 ± 1.0
	RE (%)	-0.1	6.3	5.6	-0.1
	CV (%)	10.7	6.0	4.1	1.6
Interday (n=18)					
	Mean ± SD	0.3 ± 0.0	0.6 ± 0.1	6.3 ± 0.3	61.7 ± 2.1
	RE (%)	5.4	4.6	4.9	2.8
	CV (%)	8.3	7.8	4.9	3.4

Recovery and matrix effect

The recoveries of cycloserine and IS from plasma were listed in Table 2. The mean recovery of cycloserine ranged from 97.5% to 100.4% and the mean recovery of IS was 108.5%. Assessment of the matrix effect was performed to ensure that the accuracy and precision were not affected by different batches of plasma. The back-calculated concentrations of analyte in the QCL and QCH showed an accuracy within 10.7% and a precision of less than 10.0%. These results were well within the acceptable limits and indicated no impact of the matrix effect on the analytical method.

Table 2. Recoveries of cylcoserine and IS in human plasma.

Compound	Concentration(µg/mL)	Recovery (%)
Cycloserine	0.6	100.0 ± 9.9
	6.0	100.4 ± 1.7
	60.0	97.5 ± 2.0
IS	10.0	108.5 ± 3.4

Dilution integrity

The purpose of the dilution integrity experiment was to demonstrate the validity of the dilution procedure when performing a routine analysis of study samples originally having concentrations above the ULOQ. Analysis of the dilution integrity samples after 2-fold and 4-fold dilution of QC samples that contained 120 µg/mL of cycloserine showed a %RE of less than 4.5%, whereas the %CV was less than 4.3%. These results demonstrated that the accuracy and precision, for dilution factors of 2 and 4 were acceptable per the acceptance criteria.

Stability

Stability testing was conducted to evaluate the analyte stability in stock solutions and plasma samples under different conditions. The stock solution stability was determined by comparing the peak area response of the sample of analyte and IS with that of a sample prepared from fresh stock solutions. Stock solutions of both analyte and IS maintained at room temperature were stable for 24 h, and those stored at -20°C were stable for one month. Spiked plasma samples were stable for at least 8 h at room temperature, four months at -80°C, and after three freeze-and-thaw cycles. Processed samples were stable for 14 h at 10°C in an autosampler. The RE was in the range of -9.1 to 5.7% for the QCL and -9.5 to 3.2% for the QCH. These results showed the reliable stability behavior of cycloserine under various storage conditions.

Method application

The method was applied to a pharmacokinetic study of cycloserine capsules in 11 healthy Thai volunteers. The method was sufficiently sensitive to measure plasma concentrations for up to 72 h. Three hundred seventy-four study samples were successfully analyzed for cycloserine, which demonstrated the suitability of the present method for pharmacokinetic studies. Figure 3 illustrates the mean plasma concentration-time profiles of cycloserine following the oral administration of a single dose of 250 and 750 mg capsules. The corresponding pharmacokinetic parameters of cycloserine are summarized in Table 3. There was no statistically significant difference in the half-life among the two doses applied.

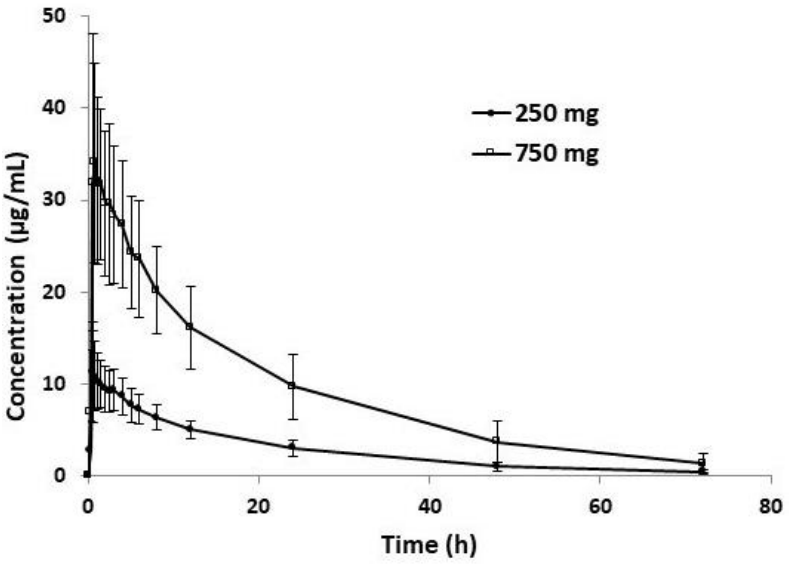

Figure 3. Mean plasma concentration-time profiles after a single-dose administration of cycloserine capsule in healthy volunteers.

Table 3. Main pharmacokinetic parameters of cycloserine after oral administration of a single dose in healthy Thai volunteers. (mean ± SD, n=11)

Parameter	250 mg	750 mg
Cmax (µg/mL)	12.5 ± 4.1	39.7 ± 10.7
Tmax (h)	0.8 ± 0.4	1.1 ± 0.9
AUC_{0-72} (µg*h/mL)	196.4 ± 39.9	633.9 ± 182.4
$AUC_{0-\infty}$ (µg*h/mL)	209.9 ± 45.1	673.5 ± 208.3
Half-life (h)	16.4 ± 4.6	16.3 ± 5.0

Discussion

The optimization of chromatographic conditions and sample preparation play an important role in the successful development of bioanalytical methods. Various columns are tested to have an acceptable chromatographic selectivity and sensitivity. The length of the columns ranged from 100 mm to 150 mm with a particle size variation of 1.7 µm to 3 µm and different stationary phases (amide, C18). After optimization, the Acclaim™ 120, C18, 2.1x100 mm, 3 µm was finalized to yield acceptable chromatography. During the selection of the mobile phase, the effects of the buffer composition, strength, various organic solvents and pH on chromatography were studied. Based on peak characterization and signal intensity, 0.1% formic acid and methanol in the ratio of 30:70 with the flow rate of 0.15 mL/min was selected.

In terms of sample preparation, protein precipitation and solid-phase extraction (SPE) were reported for the quantification of cycloserine in human plasma using LC-MS/MS methods. SPE seems to feasibly remove proteins from plasma samples; however, it requires considerable efforts for method development to optimize an SPE procedure. Protein precipitation is widely used due to its

simplicity and speediness even though it does not result in very clean extracts. Sample dilution appeared to be another approach to reduce matrix effects by enabling the introduction of less matrix components into the LC-MS/MS system (Mao *et al*, 2017). After protein precipitation, the sample supernatant was further diluted with 50% methanol using several different dilution ratios (1:1, 2:1, and 4:1, v/v). A dilution ratio of 4:1 was found to be sufficient to eliminate the matrix effects while maintaining an acceptable level of sensitivity.

The described chromatographic conditions provided a good separation between cycloserine and IS. The method proved to be specific for routine sample analyses because no interfering peaks from endogenous substances from the plasma of 11 volunteers were observed. The calibration curves were linear over the concentration ranges found clinically. The stability results of cycloserine in human plasma and in stock solutions indicated acceptable stability under various storage conditions. The validated method was successfully applied to a pharmacokinetic study of cycloserine following a single dose administration of 250 and 750 mg capsules in healthy Thai volunteers. The pharmacokinetic parameters of cycloserine obtained from the Thai subjects in this study were comparable to previous reports (Zhou *et al*, 2015; Mao *et al*, 2017).

Conclusion

An LC-MS/MS method for the determination of cycloserine in human plasma was developed and fully validated using protein precipitation coupled with dilution techniques. The method is accurate, precise, and linear over the concentration range of 0.3-70 µg/mL. The present method was successfully applied to a pharmacokinetic study of cycloserine in 11 healthy Thai volunteers after oral administration of a single dose of cycloserine at 250 and 750 mg under fasting conditions.

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