IJPSR (2020), Volume 11, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 01 November 2019; received in revised form, 20 December 2019; accepted, 25 December 2019; published 01 January 2020

SENSITIVE AND RAPID ESTIMATION OF SARECYCLINE, AN ANTIBIOTIC DRUG IN SPIKED HUMAN PLASMA BY LC-MS/MS

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Keywords:

LC-ESI-MS/MS, Sarecycline, Minocycline, Human plasma, Internal standard

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ABSTRACT: Sarecycline, a narrow spectrum tetracycline-derived antibiotic, is used to treat moderate to severe *Acne vulgaris*. The current study is aimed at developing a simple, sensitive and accurate liquid chromatography-tandem mass spectrometric (LC/MS/MS) method and validating the same for determination of sarecycline in human plasma. Zorbax, SB C18, 4.6 mm × 75 mm, 3.5 µm, 80 Å column, 10 mM ammonium formate (pH 4.5.): acetonitrile (50:50 v/v) mobile phase was used for chromatographic separation of sarecycline. The sarecycline and minocycline were monitored by electrospray ionization in positive ion multiple reaction monitoring (MRM) modes. This mode was used to detect the sarecycline and minocycline (IS) at m/z 488.6 / 291.1 and 458.4 / 337.2. Liquid-liquid extraction was employed in the extraction of analytes from human plasma. This method is validated over a linear concentration range of 0.5-150.0 ng/mL with a correlation coefficient (r) of ≥ 0.9997. Both drug and internal standards (IS) were stable in plasma samples.

INTRODUCTION: Sarecycline is the first narrow-spectrum tetracycline-class antibiotic being developed for acne treatment. In addition to exhibiting activity against important skin/soft tissue pathogens, sarecycline exhibits targeted antibacterial activity against clinical isolates of *Cutibacterium acnes* [1-3]. The antimicrobial action of tetracyclines against *C. acnes* occurs *via* inhibition of protein synthesis [4-8]. In addition, *C. acnes* also produce proteins/enzymes that play an important role in inflammation (*e.g.*, lipase) which would also be down-regulated as a consequence of inhibition of protein synthesis and may account for the anti-inflammatory properties observed with sarecycline and other tetracyclines [9-12].

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DOI: 10.13040/IJPSR.0975-8232.11(1).365-70

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(1).365-70

The chemical name of sarecycline is (4S, 4aS, 5aR, 12aR) - 4 - (dimethylamino) -1, 10, 11, 12 a-tetrahydroxy-7-[methoxy (methyl) amino] methyl] - 3, 12- dioxo - 4a, 5, 5a, 6-tetrahydro-4H-tetracene-2-carboxamide **Fig. 1**.

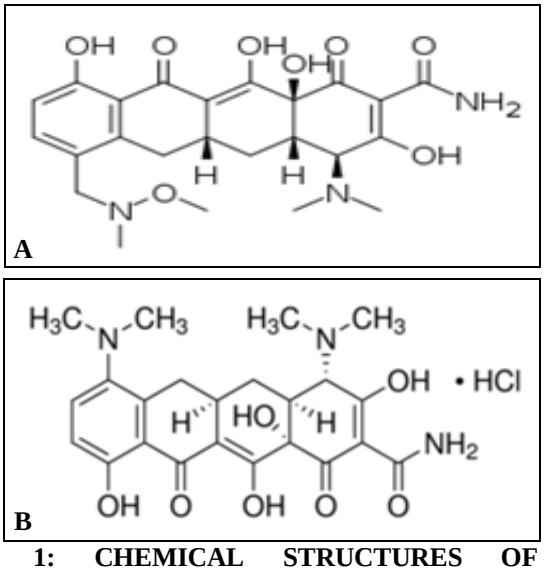

FIG. 1: CHEMICAL STRUCTURES OF A) SARECYCLINE B) MINOCYCLINE

The molecular formula is $C_{24}H_{29}N_3O_8$ and the molecular weight is 487.51. The literature survey reveals that one method was reported for quantification of sarecycline by using LC-MS/MS [13-17]. There is no method reported for the estimation of sarecycline using internal standard in biological samples.

The main goal of the present study is to develop and validate the novel, simple, sensitive, selective, rapid, rugged and reproducible analytical method for the quantitative determination of Sarecycline in human plasma by HPLC-ESI-MS/MS [18-22].

MATERIALS AND METHODS:

Chemicals and Reagents: Sarecycline HCl and Minocycline HCl were obtained from clear synth Labs (P) Ltd, Mumbai, India. Human plasma (K2EDTA), obtained from Navjeevan blood bank, Hyderabad. Formic acid, Ammonium formate, sodium carbonate, acetonitrile, methanol obtained from SD- Fine Chemicals, Mumbai. MTBE (methyl tertiary butyl ether) was obtained from Labscan, Mumbai. (Ultra pure water obtained from Milli-Q System).

Instrumentation: The 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. Data processing was performed on the Analyst 1.4.1 software package (SCIEX).

Detection: The mass transitions were selected as m/z 488.6 / 291.1 and 458.4 / 337.2 for quantification of SC and MC respectively **Fig 2** and **3**.

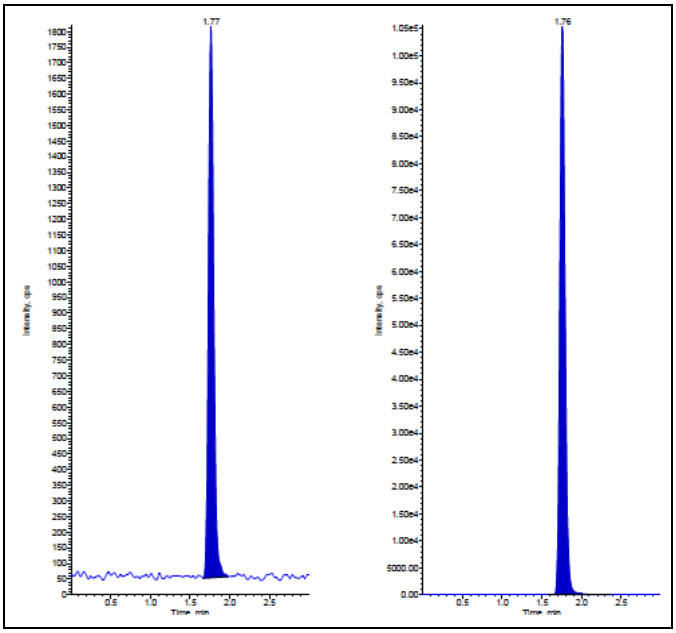

FIG. 2: REPRESENTATIVE CHROMATOGRAMS OF SARECYCLINE (SC) & MINOCYCLINE (MC) IN HUMAN PLASMA

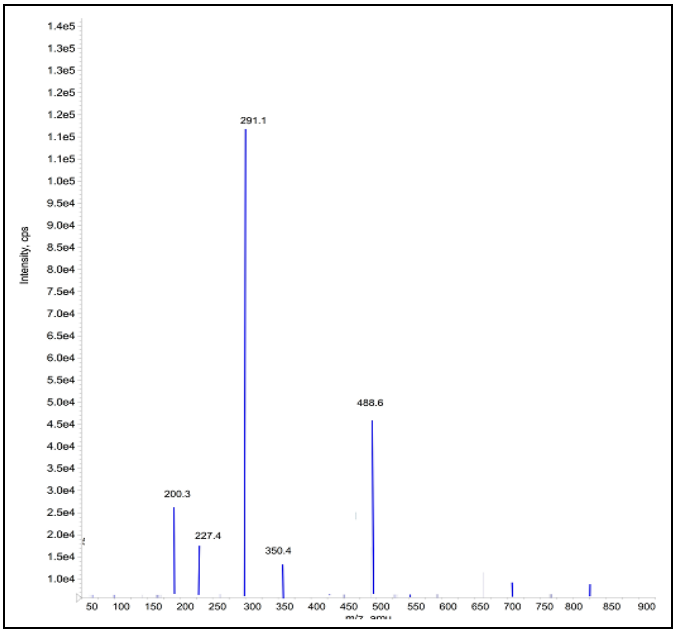

FIG. 3: MASS SPECTRUM OF SARECYCLINE Q1 AND Q3 IONS

Chromatographic Conditions: A good separation and elution were achieved using Zorbax, SB C_{18}, 4.6 mm × 75 mm, 3.5 µm was selected as the analytical column. The mobile phase composition was 10mM Ammonium formate: acteonitrile (50:50 v/v) at a flow rate of 0.5 mL/min and 10 mL injection volume was used. The column temperature was set at 40 °C. Minocycline was found to be appropriate internal standard. Retention time of sarecycline and minocycline were found to be 1.5 ± 0.2 min, with overall runtime of 3.0 min.

Calibration Standards and Quality Control Samples: Standard stock solutions of sarecycline (100.0 µg/mL) and minocycline (100.0 µg/mL) were prepared in methanol. The spiking solution for Minocycline (80.0 ng/mL) was prepared in 50% methanol from a respective standard stock solution. Standard stock solutions and IS spiking solutions were stored in the refrigerator (2-8 °C) until analysis. Standard stock solutions were added to drug-free human plasma to obtain sarecycline concentration levels of 0.5, 1.0, 5.0, 15.0, 30.0,

45.0, 60.0, 90.0, 120.0 and 150.0 ng/mL for analytical standards and 0.5, 1.5, 75.0 and 105.0 ng/mL for quality control standards and stored in a -30 °C setpoint freezer until analysis. The aqueous standards were prepared in reconstitution solution (10 mM ammonium formate: acetonitrile (50:50 v/v) for validation exercises until analysis.

Sample Preparation: Liquid-liquid extraction was used to isolate sarecycline and minocycline from human plasma. 100 µL of minocycline spiking solution (8.0 ng/mL) and 200 µL of plasma sample (respective concentration) were added respective ria vials and vortexed for 30 seconds followed by 100 µl of 0.5N sodium carbonate solution was added and vortexed for 10 minutes. Then 2.5 mL of extraction solvent (Methyl Tertiary butyl ether) was added and vortexed approximately for 20 min. This was followed by centrifugation at 4000 rpm for 5 min at 20 °C. Then samples were Flash freeze by using dry-ice/Acetone. The supernatant from each ria vial was transferred into another set of fresh ria vial. These samples were evaporated at 40 °C under nitrogen until dry. Finally, the dried residue samples were reconstituted with 500 µL of reconstitution solution (10mM Ammonium formate (pH 4.5): acetonitrile 50:50, v/v) and vortexed briefly. These samples were transferred into autosampler vials and injected into LC-MS/MS.

Selectivity and Specificity: Selectivity was performed by analyzed the human blank plasma samples from six different sources (donors) with an additional hemolysed group and lipedimic group to test for interference at the retention times of analytes. The peak area of SC in blank samples should not be more than 20% of the mean peak area of LOQ of SC. Similarly, the peak area of MC in a blank sample should not be more than 5% of the mean peak area of LOQ of MC.

Precision and Accuracy: Precision and accuracy were determined by replicate analysis of quality control samples (n = 6) at LQC (low-quality control), MQC (medium quality control) and HQC (high-quality control) levels. The % CV should be less than 15% and accuracy should be within 15% except for LLOQ where it should be within 20%.

Matrix Effect: The matrix effect due to plasma was used to evaluate the ion suppression/ enhancement in a signal when comparing the absolute response of QC samples after pretreatment (Liquid-liquid extraction with MTBE) with that of the reconstituted samples. Experiments were performed at MQC levels in triplicate with six different plasma lots. The acceptable precision (% CV) of ≤ 15% was maintained.

Recovery: The extraction efficiencies of SC and MC were determined by analysis of six replicates at each quality control concentration level and at one concentration for the internal standard MC. The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of non extracted standards (spiked into the mobile phase).

Limit of Detection and Quantification (LOD and LOQ): The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples. The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of MC.

Stability (Freeze-Thaw, Autosampler, Room Temperature, Long Term): Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from the fresh stock solution.

Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. The analyte was considered stable if the % Change is less than 15% as per US FDA guidelines [18-20]. The stability of spiked human plasma samples stored at room temperature (benchtop stability) was evaluated for 26 h. The stability of spiked human plasma samples stored at -30 °C in autosampler (autosampler stability) was evaluated for 55.5 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately

(time 0 h), with the samples that were reinjected after storing in the autosampler at 20 °C for 26 h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the autosampler at 20 °C for 27 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at –30 °C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long term stability evaluation, the concentrations obtained after 65 days were compared with initial concentrations.

RESULTS AND DISCUSSION:

Method Development and Validation: The goal of this work is to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of MC from plasma samples. LC-MS/MS has been used as one of the most powerful analytical tools in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The MS optimization was performed by direct infusion of solutions of SC and MC into the ESI source of the mass spectrometer. The vital parameters like ionization type, temperature and voltage, gas parameters such as nebulizer and heater gases, compound parameters like DP, EP, FP, CE and CXP were optimized to obtain a better spray shape and ionization to form the respective productions from the protonated SC and MC molecules. Chromatographic conditions, especially the composition of the mobile phase and selection of suitable column were optimized through several trials to achieve the best resolution and increase the signal of analyte and internal standard. Different extraction methods like solid-phase extraction, liquid-liquid extraction, precipitation methods were optimized for extraction of SC and MC from the plasma sample. Good separation and elution were achieved using 5 mM ammonium formate: acetonitrile (10:90 v/v) as the mobile phase, flow-rate of 0.5 mL/min and injection volume of 10 µL. Liquid-liquid extraction was chosen to optimize the drug and internal standard. Minocycline was found to be an appropriate internal standard. The retention time of sarecycline and minocycline were found to be 1.5 ± 0.2 min, with an overall runtime of 3.0 min **Fig. 4**.

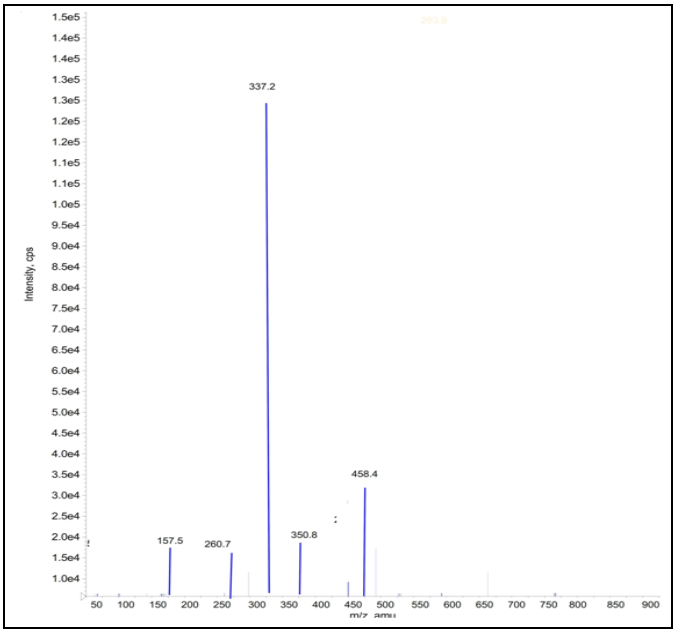

FIG. 4: MASS SPECTRUM OF MINOCYCLINE OF Q1 AND Q3 IONS

Linearity: Calibration curve was plotted as the peak area ratio (SC/MC) versus (SC) concentration. Calibration was found to be linear over the concentration range of 0.5 - 150.0 ng/mL. The correlation coefficient (r^2) was greater than 0.9997 for all curves in **Table 1**.

TABLE 1: CALIBRATION CURVE DETAILS OF SARECYCLINE

Spiked plasma concentration (pg/mL)	Concentration measured (mean) (pg/mL) (*n* = 5)	Precision RSD (*n* = 5)	Accuracy %
0.5	0.51	1.1	102.00
1.0	.93	4.4	96.60
1.5	1.46	5.1	99.68
5.0	4.8	1.7	100.00
15	15.6	3.3	100.65
30	30.5	5.2	100.21
45	45.2	3.2	99.28
60	60.4	2.5	99.40
90	90.12	4.7	103.17
120	120.5	3.2	98.43
150	150.4	1.7	99.12

Selectivity: The selectivity of the method assessed by comparing the chromatograms obtained. There were no significant endogenous peaks observed at the respective retention time of SC and MC. The results indicate that the method exhibited both good specificity and selectivity.

Precision and Accuracy: Precision and accuracy for this method were controlled by calculating the Within-run and Between-run variations at three

concentrations (1.5, 75, 105 ng/mL) of QC samples in six replicates. As shown in **Table 2**. The within-run precision and accuracy were between 0.79 to 3.05 and 98.67 to 103.87 % for MC. Similarly, the Between-run Precision and Accuracy were between 1.58 to 5.20 and 96.90 to 103.8 % for SC. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

TABLE 2: PRECISION AND ACCURACY (ANALYSIS WITH SPIKED PLASMA SAMPLES AT THREE DIFFERENT CONCENTRATIONS)

Spiked plasma concentration (pg/mL)	Within-run (n=6)			Between-run (n=6)		
	Concentration measured (ng/mL) (mean)	Precision RSD	Accuracy %	Concentration measured (ng/mL) (mean)	Precision RSD	Accuracy %
0.5	0.54	1.1	102.7	0.52	3.8	110.6
1.5	1.52	2.1	105.5	1.48	3.6	105.2
75	75.3	2.7	103.2	75.2	3.5	104.3
105	105.7	1.5	91.7	105.4	3.8	103.9

Matrix Effect: The ion suppression/enhancement in the signal at MQC level was found % CV less than 5. These results indicating that there is no effect on ion suppression and ion enhancement.

Recovery: The % recoveries of RC were determined at three different concentrations 1.5, 75 and 105 ng/mL, which was found to be 94.18, 98.33 and 99.36%. The overall average recoveries of SC and MC were found to be 94.12%. Recoveries of the analyte and IS were consistent, precise and reproducible.

Limits of Detection and Quantification (LOD & LOQ): The LOQ& LOD signal-to-noise (S/N) values found for six injections of SC at LOQ & LOD concentrations were 0.5 ng/ml and 0.05 ng/ml.

Stability (Freeze-Thaw, Autosampler, Room Temperature, Long Term): Stock solution stability was performed to check the stability of SC and MC in-stock solutions prepared in methanol and stored at 2-8 °C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 26 days. The % change for SC and MC was less than 1% indicates that stock solutions were stable at least for 26 days. Room temperature and autosampler stability for SC were investigated at LQC and HQC levels. The results revealed that SC was stable in plasma for at least 26 h at room temperature, and 57 h in an autosampler. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with SC at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that SC was stable in a matrix up to 65 days at a storage temperature of -30 °C.

The results obtained from all these stability studies are tabulated in **Table 3**. Precision (% CV) is less than 5 for Room temperature, long-term, Freeze-thaw, autosampler stability.

TABLE 3: STABILITY OF SARECYCLINE IN HUMAN PLASMA SAMPLES

Stability experiments	Storage condition	Spiked plasma concentration (pg/ml)	Concentration measured (*n*=6) Mean	RSD (*n*=6) (%)	Accuracy (%)
Bench top in Plasma	RT	1.5	1.54	1.2	98.9
	26 h	105	6105.21	3.3	81.5
Processed	Autosampler	1.5	1.53	1.4	108.2
(extracted sample)	57 h	105	105.22	1.5	90.4
Freeze/Thaw stability	-30 °C	1.5	156.5	2.5	104.3
	Cycle-3	105	7381.7	2.6	90.4
Long-term stability in	- 30 °C	1.5	160.3	8.4	106.9
human plasma	65 days	105	7450.0	3.1	90.5

CONCLUSION: The proposed research work is highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other described methods in previously. Quantification of sarecycline was compared with respective isotope labeled internal standards. Extraction of analyte and IS were achieved by using LLE. Linearity range, column,

mobile phase, flow rate, injection volume, plasma usage volume for analysis was improved. Hence this method has significant advantages over previously reported methods in-terms of Selectivity, sensitivity, Linearity, Reproducibility.

ACKNOWLEDGEMENT: Authors wish to thank the support received from IICT (Indian Institute of Chemical Technology), Hyderabad, India for providing a literature survey and APL Research Pvt. Ltd. Hyderabad, India to carry out this research work.

CONFLICTS OF INTEREST: Authors declare that there is no conflict of interest.

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How to cite this article:
Prasad BS and Kumari SJ: Sensitive and rapid estimation of sarecycline, an antibiotic drug in spiked human plasma by LC-MS/MS. Int J Pharm Sci & Res 2020; 11(1): 365-70. doi: 10.13040/IJPSR.0975-8232.11(1).365-70.