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Method development, validation, and application of liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry method for the quantification of amprenavir in plasma samples

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ABSTRACT

A specific liquid chromatography-mass spectrometry/mass spectrometry spectrometric procedure for the quantitation of amprenavir drug in biological matrices was developed and validated. Chromatographic isolation was accomplished through a Zorbax C18 analytical stationary phase having the dimensions of 50 mm × 4.6 mm and particle size of 5.0 µm. Isocratic separation was processed with acetonitrile 0.1%v/v HCOOH in water and methyl alcohol in the proportion of 60:10:30 as a moveable system with a flow rate of 0.60 mL/min. Liquid–liquid extraction was carried out for drug and internal standard isolation with an ethyl acetate solvent. Parent and product ionic components were examined at m/z 506.2 → 89.1 for amprenavir and 367.1 → 350.1 for rilpivirine internal standard on the MRM (multiple reaction monitoring) mode. The linearity plot of analyte was rectilinear in the concentration over 0.15–1500 ng/mL with the correlation coefficient value of r^2 being >0.990. %relative standard deviation findings were <4.21% for intraday and interday accuracy and precision. The technique has good recoveries, and %recovery findings of LQC (low quality control), MQC(median quality control), and HQC (high quality control) solutions were 92.9%, 95.1%, and 96.4%, respectively. Amprenavir has more stability for longer time when subjected to different stability environments and the procedure was efficiently relevant to the regular investigation of amprenavir analyte in the biological matrix.

INTRODUCTION

Amprenavir is chemically represented as (3*S*)-oxolan-3yl-*N*[(2*S*, 3*R*)-3- hydroxy-4-[*N*-(2-methyl propyl) (4-amino benzene) sulfonamido]-1-phenylbutan- 2-yl]carbamate having molecular weight and formula of 505.628 g/mol and $C_{25}H_{35}N_3O_6S$, respectively (Fig. 1) (Sadler *et al*., 1999; Wittayanarakul *et al*., 2008). The human immunodeficiency virus (HIV) and its clinical syndrome AIDS continue to be main health issues around the universe. The extremely improved and effective chemotherapy (antiretroviral) for AIDS is mostly used in curbing the disease during the pandemic (Dandache *et al*., 2007; Zhou *et al*., 2009). This drug inhibits the proteases with the action against the HIV-I. The compounds which act against the proteases inhibit a part (protease) of HIV. HIV-I protease is a kind of enzyme which requires a cleavage of viral polyprotein (proteolytics) precursors into the individual proteins (functional) found in contagious HIV-I. The drug binds to the main site of protease and prevents enzyme action. This action prevents the cleaving of viral polyprotein components, resulting in development of juvenile noninfectious viral element components. Protease inhibitor components are nearly always useful in combination with other two anti-HIV compounds (Brophy *et al*., 2000; Granfors *et al*., 2006).

The literature on amprenavir unveiled that there are few analytical procedures available for the estimation of drug in API

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and dosage forms on liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) with more retention times. Few reported methods were on spectroscopy (Padmini *et al*., 2017), liquid chromatography (Rajitha *et al*., 2014), and LC-MS/MS (Jingduan *et al*., 2002; Samson *et al*., 2015). The development of specific technique like LC-MS/MS is greatly needed for the quantification of amprenavir in biological matrix samples. Thereby, the method is applicable for the analysis of different types of bio-samples having amprenavir and can perform the bioavailability, bioequivalence, and forensic studies.

MATERIALS AND METHODS

Chemicals and reagents

Amprenavir (99.89% pure) was acquired from MSN Labs, Hyderabad, India. Rilpivirine internal standard of 99.91% was received from Hetero Labs, Hyderabad, India. Acetonitrile (ACN) and methanol of LC grade and HCOOH of analytical grade were procured from JT Bakers, Ahmedabad, India.

Liquid chromatographic MS/MS system

An LC-MS/MS SCIEX API4000 instrument, furnished with a positive electrospray ionization source and HPLC of Shimadzu, consisted of an SIL-HTC autosampler, dual pump, and column oven, which were utilized in the present work. Analyte quantitation, acquisition of data, and its integration were processed by utilizing Analyst Software version 1.6.3.

Chromatographic conditions

Chromatographic isolation was accomplished through a Zorbax C18 analytical stationary phase having the dimensions of 50 × 4.6 mm and particle size of 5.0 µm. Isocratic separation was processed with ACN 0.1%v/v HCOOH in water and methyl alcohol in the proportion of 60:10:30 as a moveable system with a flow rate of 0.60 ml/minute. Volume of the injection was 5.0 µl. Amprenavir and rilpivirine (IS) were isolated with a total runtime of 6 minutes. The temperature of the autosampler and stationary phase were supervised at 5°C and 30°C, correspondingly.

MS/MS system conditions

The mass system functioned via the MRM mode with a positive ion approach for both amprenavir and IS. The adjusted mass system parameters for amprenavir and IS were as follows: both drying gas and sheath gas temperatures were 300°C; nebulizer pressure was monitored at 22.0 psi; sheath gas and drying gas flow rates were 10 L/min and 5L/min, respectively; capillary voltage was set at 3 kV; Collision energy and fragmentor voltage were 15 eV and 110 V for amprenavir and 15 eV and 115 V for IS; parent-to-product ion conversions examined were *m/z* 506.2 → 89.1 for amprenavir and 367.1 → 350.1 for rilpivirine; and for every transition, the dwell timing was set to 200 ms.

Calibration curve standard solutions

Exactly 1000 µg/ml stock solution of amprenavir was executed freshly by dissolving 10 mg of analyte in 10 ml of 70.0% ACN. The calibration standard concentrations of eight dissimilar levels were prepared by method of spiking to plasma blank with amprenavir standard to acquire 0.15, 30.0, 125.0, 275.0, 500.0, 800.0, 1,150.0, and 1,500.0 ng/ml concentrations.

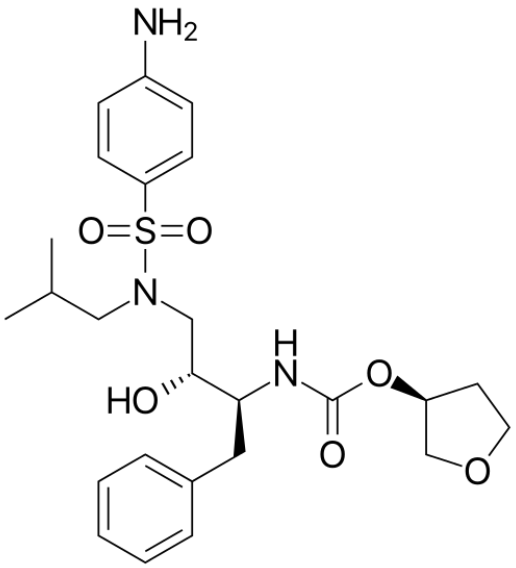

Figure 1. Amprenavir chemical structure.

Quality control standard solutions

The standards were prepared at three dissimilar concentrations of HQC, MQC, and LQC standards. These quality control (QC) levels were prepared as per the calibration standard solutions to acquire 1125.0, 750.0, and 0.42 ng/ml concentrations for HQC, MQC, and LQC, correspondingly. The processed samples were stored at −20.0°C up to the analysis time.

Sample processing technique

Analyte solution was processed by relocating 250.0 µl of plasma and 50 µl of rilpivirine (1 µg/ml) into a propylene tubes and vortexed for 2 minutes. Amprenavir and IS were separated with 5.0 ml ethyl acetate solvent and processed for centrifugation at 3,500 rpm for 30 minutes. Furthermore, the organic portion was isolated and subjected to drying in a lyophiliser. The residue was dissolved in 250 µl of moveable solvent and then translocated into pre-labeled tubes.

Method validation

The developed process was subjected to validation and the parameters were selectivity, specificity, matrix effect, stability, linearity, precision, accuracy, and recovery (EMA, 2011; ICH, 2005; USFDA, 2001).

RESULTS AND DISCUSSION

Mass spectrometry instrument parameters

The product ion mass scale of amprenavir and relpivirine obtained at *m/z* 89.1 and 350.1 were opted as detecting ions. Meanwhile, the mass scale parameters collision and curtain gas,

ionspray voltage temperature, capillary voltage, nebulizer, and heater gas were improved to attain maximum mass spectrum response.

Internal standard selection

In the present work, rilpivirine was chosen as an internal standard because of its parallel chromatographic performance, extraction efficiency, ionization, and retention activities as the amprenavir; there was no noticeable interference during retention timings of analyte and rilpivirine in accordance with findings of method validation (Chambers *et al*., 2014; Henion *et al*., 1998).

Method validation

Specificity

Plasma blanks gained from six dissimilar lots of plasma samples were spiked with analyte drug at lower limit of quantification (LLOQQC) and relpivirine to evaluate specificity. In the second figure (Fig. 2), the retention times of amprenavir and relpivirine were 1.2 and 2.3 minutes, correspondingly. No interferences were observed for matrix substances at the retention times of the drug and internal standard.

Calibration curve and sensitivity

The calibration curve was plotted for amprenavir and excellent results (Table 1) were shown in calibration limits of 0.15–1,500.0 ng/ml. Rectilinear graph was formed by the peak response fractions (y) of amprenavir to internal standard versus concentration levels (x) with the weighting factor ($1/X^2$) (Murphy *et al*., 1995; Patel *et al*., 2011). Developed process regression formula of linearity curve was $y = 0.00258x + 0.00714$ with a regression coefficient (r^2) value of 0.9993. The analyte LLOQ was 0.15 ng/ml, evaluated by five replicate analyte solutions having more than three signal-to-noise ratio values.

Accuracy and precision

Intrabatch and interbatch precision and accuracy were executed by six spiked (amprenavir) plasma samples at HQC, MQC, LQC, and LLOQ concentrations in a lot and in three succeeding lots, correspondingly (Cha *et al*., 2020; Elawadya *et al*., 2020). Table 2 shows amprenavir findings of for accuracy and precision. %relative standard deviation (RSD) findings of intrabatch and interbatch precisions were within the limits and the findings were between 1.86 and 4.21. The relative error findings of intrabatch and interbatch accuracy were in the limits of −4.43 to 6.15.

Recovery

The recoveries (extraction) were calculated by calculating six responses of the peak ratios of HQC, MQC, and LQC level solutions of amprenavir to spiked sample solutions after extraction at respective concentration levels (El-Zaher *et al*., 2019; Singh *et al*., 2020). In the same manner, extraction recoveries of rilpivirine were calculated by calculating the peak area ratios of quality control plasma sample solutions ($n = 6$) to spiked human plasma samples at respective concentration levels. The average extraction recoveries of amprenavir were 96.4%, 95.1%, and 92.9% at high, medium, and low QC points, correspondingly. The average extraction recovery of rilpivirine was 95.9% at the 100 ng/ml concentration level (Figs. 3–5 and Table 3).

Matrix factor

Matrix constituents will hike or suppress the ionization process. Its effect was evaluated by determining the internal standard normalized matrix factor (MF) at eight variable lots (having two lipemic and two hemolytic lots) of plasma samples (Logoyda, 2020). The mean internal standard normalized MF for all the analytes was in the limits of 1.04–0.94. Table 4 shows the MF %RSD findings, which is ≤3.57.

Integrity of dilution

Integrity dilution was executed at twofold concentrations of the upper limit of quantification (ULOQQC) for amprenavir. After dilution in the proportion of 1:4, the mean back calculated analyte amount for dilution QC sample solutions were in limits of 85.0%–115.0% of original figure with %RSD of ≤3.86.

Stability

Amprenavir stability was processed in both aqueous and matrix-based sample solutions. Amprenavir and rilpivirine were not affected in stock levels monitored for 70 days at 1.0–10.0°C. Stocks in the diluent for 48 hours at 1.0°C–10.0°C were not

Table 1. Amprenavir calibration standard concentrations.

ID	Concentration (ng/ml)	Mean (ng/ml)	%RSD	%RE
CS-1	0.15	0.144	3.8	4
CS-2	30	28.89	2.7	3.7
CS-3	125	123.64	2.1	1.088
CS-4	275	269.19	4.2	2.11
CS-5	500	487.99	3.5	2.40
CS-6	800	786.71	1.9	1.66
CS-7	1150	1098.2	2.9	4.50
CS-8	1500	1479.52	3.7	1.36

RSD: Relative standard deviation; RE: Relative error.

Table 2. Interbatch and intrabatch accuracy and precision.

Concentration level	Nominal concentration (ng/ml)	Intrabatch			Interbatch		
		Quantity found (ng/ml)	%RSD	%RE	Quantity found (ng/ml)	%RSD	%RE
LLOQ	0.15	0.1433	2.92	−4.43	0.1467	1.86	−2.22
LQC	0.42	0.4352	4.21	3.62	0.446	2.47	6.15
MQC	750	770.79	3.43	2.77	779.935	2.94	3.99
HQC	1125	1131.49	4.16	0.58	1191.10	4.19	5.87

RSD: Relative standard deviation; RE: Relative error.

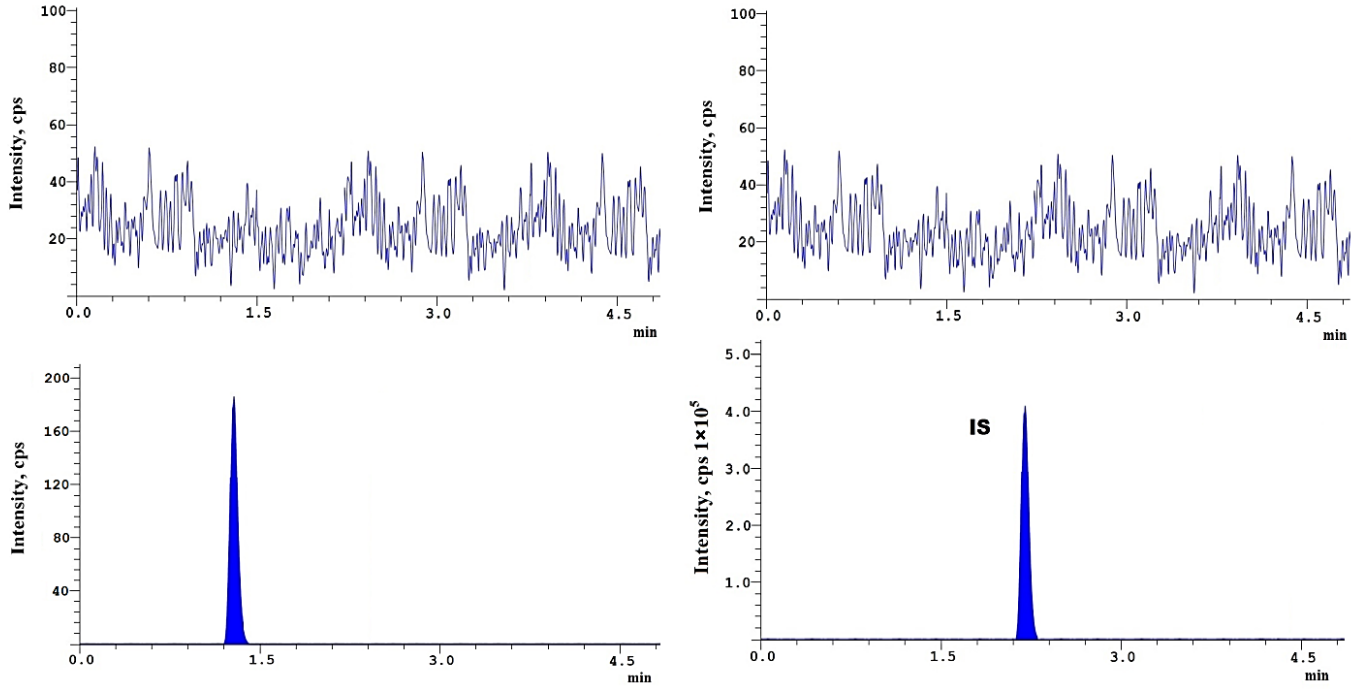

Figure 2. Amprenavir (a) blank plasma and (b) LLOQC sample chromatograms. IS: Internal standard; ACN, 0.1%v/v HCOOH in water and methyl alcohol in the proportion of 60:10:30; flow rate of 0.60 ml/minute.

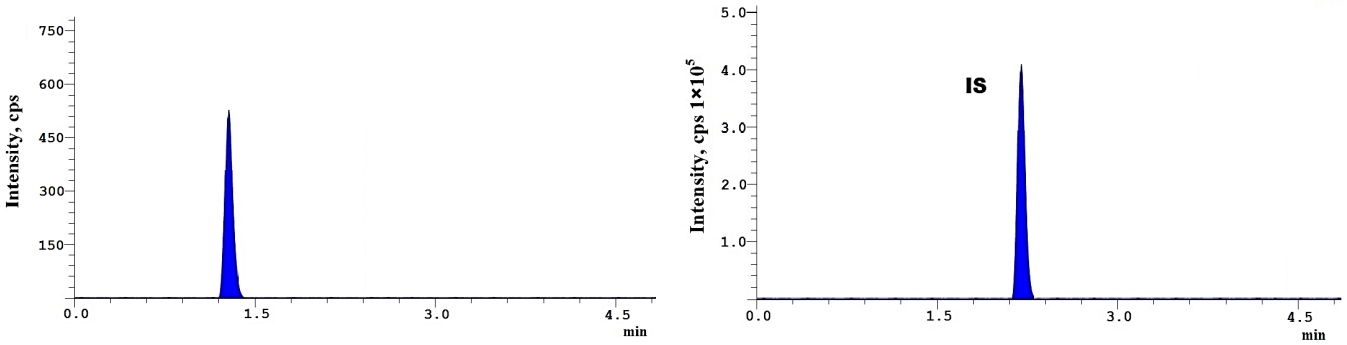

Figure 3. Amprenavir chromatogram at LQC. IS: Internal standard; ACN, 0.1%v/v HCOOH in water and methyl alcohol in the proportion of 60:10:30; flow rate of 0.60 ml/minute.

affected. Matrix stability was noted for 60 days at −70.0°C and −20.0°C against fresh sample solutions of linearity QCs. Stability finding are given in Table 5. No degradation was observed up to 20 hours at benchtop stability at 10.0°C and after 6 freeze-and-thaw sequences. In the autosampler, the analytes were no effected up to 72 hours, which was kept at 10.0°C.

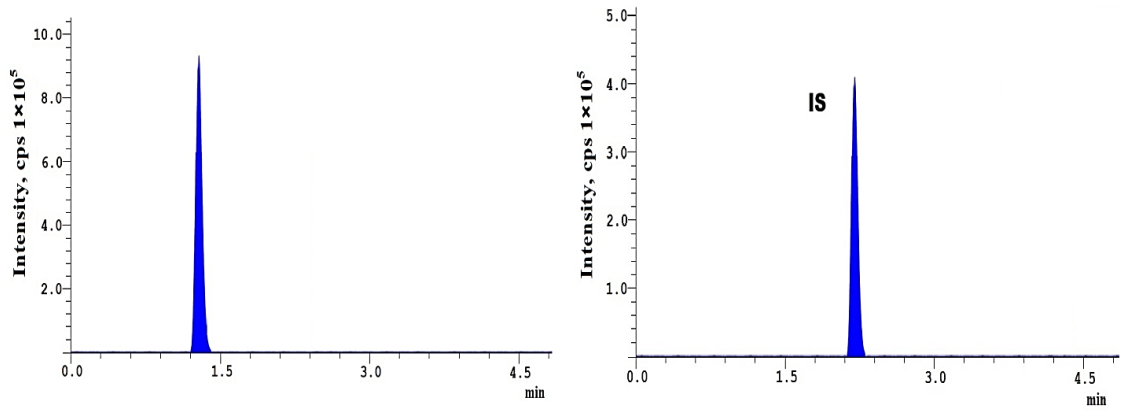

Figure 4. Amprenavir chromatogram at MQC. IS: Internal standard; ACN, 0.1%v/v HCOOH in water and methyl alcohol in the proportion of 60:10:30; flow rate of 0.60 ml/minute.

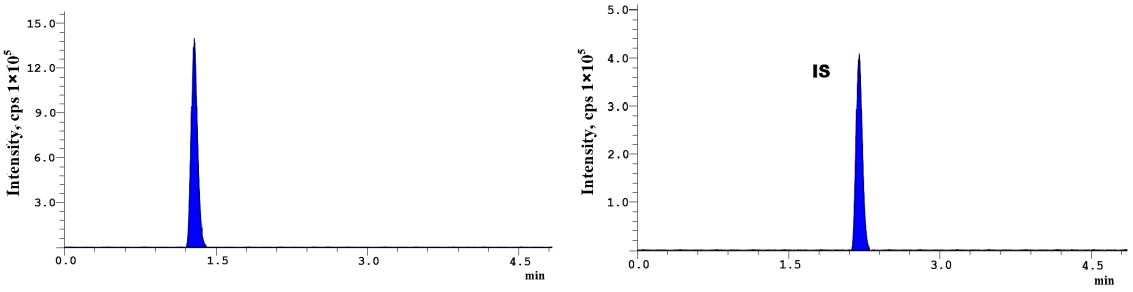

Figure 5. Amprenavir chromatogram at HQC. IS: Internal standard; ACN, 0.1%v/v HCOOH in water and methyl alcohol in the proportion of 60:10:30; flow rate of 0.60 ml/minute.

Table 3. Amprenavir and IS recoveries.

Conc.	*A*	*B*	% Recovery	%Mean recovery	%RSD
LQC	556	516	92.9	94.8	1.52
MQC	988,975	940,515	95.1		
HQC	1,484,862	1,431,406	96.4		
IS	459,326	434,522	95.9		

RSD: Relative standard deviation; *A*: mean recoveries of unextracted samples; *B*: average recovery of extracted samples; IS: internal standard.

Table 4. Amprenavir matrix effect.

Amprenavir	LQC			HQC		
	Drug MF	IS MF	IS normalized MF	Analyte MF	IS MF	IS normalized MF
L-1	1.07	1.12	0.960	1.12	1.06	1.057
L-2	1.06	1.07	0.990	1.07	1.10	0.970
L-3	1.10	1.05	1.048	1.05	1.11	0.940
L-4	1.11	1.08	1.028	1.08	1.12	0.960
L-5[a]	1.12	1.09	1.028	1.13	1.09	1.0370
L-6[a]	1.09	1.09	1.000	1.08	1.06	1.019
L-7[b]	1.11	1.08	1.030	1.09	1.10	0.990
L-8[b]	1.12	1.13	0.990	1.09	1.11	0.980
Mean	1.008			0.99		
SD	0.028			0.04		
%RSD	2.75			3.57		

[a]Hemolyzed lot.
[b]Lipemic lot.
RSD: Relative standard deviation; MF: matrix factor; IS: internal standard.

Table 5. Amprenavir stability.

Stability type	QC level	*X*	*Y*	%RSD	%Stability
Long term for 60 days (−70°C)	LQC	0.42	0.401	1.53	95.54
	HQC	1125	1,116.39	3.91	99.24
Refrigerator for 48 hours (1.0°C–10.0°C)	LQC	0.42	0.41	1.86	98.75
	HQC	1125	1,101.01	2.82	97.87
In-injector (at 10°C for 72 hours)	LQC	0.42	0.414	4.93	98.47
	HQC	1125	1,140.01	3.62	101.33
Benchtop for 20 hours (at <10°C)	LQC	0.42	0.40	3.49	96.37
	HQC	1125	1,074.11	2.83	95.48
Long term for 60 days (−20°C)	LQC	0.42	0.425	3.41	101.12
	HQC	1125	1,110.91	1.84	98.75
Freeze/thaw	LQC	0.42	0.39	3.92	94.64
	HQC	1125	1,144.08	4.14	101.69

X: nominal concentrations; *Y*: mean concentration of analyte; RSD: Relative standard deviation.

CONCLUSION

In the present study, a sensible and precise LC-MS/MS procedure was developed and validated for the successful quantification of amprenavir in human biological samples. This technique showed good linearity, accuracy, specificity, stability, and precision. The rectilinear plot equation and regression coefficient (r^2) outcomes are $y = 0.00258x + 0.00714$ and 0.9993, correspondingly. %RSD findings of interday and intraday precisions of executed method were in between 1.86% and 4.21% for QC standards (0.42, 750.0, and 1,125.0 ng/ml). Hence, the developed technique can be valid for the pharmacokinetics and toxicity studies in bioanalytical, forensic, and clinical analysis of amprenavir in variable type of bio-samples successfully.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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