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Development and Validation of a Robust LC-MS-MS Method for the Quantification of Amlodipine in Human Plasma

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Abstract

The primary objective of this study was to develop a simple, precise, rapid, environmentally friendly, and highly accurate method for the simultaneous quantification of amlodipine in human plasma. Optimal peak resolution was achieved using an Intersil ODS gum C18 column (4.6 × 50 mm, 3.5 µm). Chromatographic separation was performed in gradient mode with a mobile phase comprising acetonitrile, water, and formic acid. The mobile phase was delivered at a flow rate of 0.500 mL/min into the ESI (Electrospray Ionization) chamber of the mass spectrometer. The total runtime for chromatographic analysis was 8.5 minutes, with amlodipine eluting at approximately 5.25 minutes. A linear calibration curve was established in the range of 1 to 130 ng/mL for amlodipine in human plasma. The method's efficiency was demonstrated by the mean percentage recoveries at various quality control levels: 104.1% at Low Quality Control (LQC), 105.0% at Medium Quality Control (MQC), and 108.7% at High Quality Control (HQC). The Lower Limit of Quantification (LLOQ) was determined to be 1.01 ng/mL, with a relative standard deviation (RSD) of less than 20%. The accuracy of the LLOQ was evaluated across different biological matrices, showing a value of 100.5% for amlodipine, which is well within the acceptable range of 80.00% to 120.00% across seven different plasma batches. The method was developed as a rapid, robust approach for the quantification of amlodipine in human plasma and was validated rigorously following the guidelines set forth by the International Council for Harmonization (ICH). The results confirm that this method is both reliable and efficient, making it suitable for routine analysis of amlodipine in human plasma samples.

Keywords: Amlodipine; LC-MS-MS; Method development; Validation; Human plasma

1. Introduction

Bioanalysis plays a critical role in the drug discovery and development process. It focuses on the detection and quantification of analytes-such as drugs, their metabolites, and biomarkers-in various biological matrices. This process involves multiple key steps, ranging from the collection of biological samples to their final analysis and the reporting of data [1-2, 13, 17].

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The initial step in bioanalysis involves the collection of samples from either clinical or preclinical studies, which are then transported to the laboratory for further analysis. Once the samples arrive, the next crucial phase is sample preparation or clean-up. This step is vital for ensuring the accuracy and reliability of bioanalytical results, as it involves the removal of any matrix-related interferences that could affect the analysis. A robust and well-optimized sample preparation protocol is essential to improve the performance of the analytical system and yield dependable data. However, this stage of bioanalysis is often labor-intensive and can require significant time, as it demands careful handling to ensure the elimination of contaminants while preserving the analyte of interest [3-5, 10].

Following sample preparation, the final step is the actual analysis and detection of the analytes. In bioanalytical laboratories, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the preferred method for this purpose. LC-MS/MS is widely regarded for its high sensitivity, specificity, and ability to handle complex biological samples, making it an indispensable tool for bioanalysis. Through its powerful separation and detection capabilities, LC-MS/MS enables precise quantification of drugs, metabolites, and biomarkers, supporting reliable outcomes in drug development and related studies [5-9, 22-25].

Amlodipine, first approved by the FDA in 1987, is a widely used antihypertensive medication that belongs to the dihydropyridine class of calcium channel blockers. This class of drugs is particularly selective for peripheral blood vessels, which contributes to their favorable safety profile. Unlike other calcium channel blockers, dihydropyridines such as amlodipine are associated with a reduced risk of myocardial depression and disturbances in cardiac conduction, making them a preferred choice in certain clinical settings [10, 15-19, 27-30].

Amlodipine is frequently prescribed for the management of hypertension and angina, offering effective control of high blood pressure and relief from chest pain. One of the notable attributes of amlodipine is its antioxidant properties, along with its capacity to stimulate the production of nitric oxide (NO). Nitric oxide is a critical vasodilator that helps relax blood vessels, thereby contributing to lower blood pressure. Additionally, amlodipine's long half-life allows for convenient once-daily dosing, making it an attractive option for patients seeking a manageable and effective treatment for cardiovascular conditions. Its structure is shown in Fig. 1. Chemical name of amlodipine is 3-O-ethyl-5-O-methyl-2-(2-aminoethoxymethyl)-4-2(2-chlorophenyl)-6-methyl)-1,4-dihydropyridine-3,5-dicarboxylate [6-8, 26, 11].

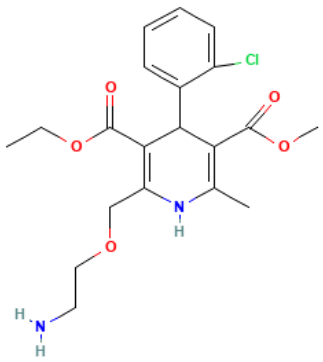

Figure 1. Chemical structure of amlodipine

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2. Materials and Methods

Chemicals and reagents

Amlodipine, with a purity of 100.0%, and tolterodine, used as an internal standard with a purity of 99.9%, were sourced from Moehs Catalana, S.L., Spain, and Zhejiang Huahai Pharmaceutical Co., Ltd, China, and certified by the EDQM – Council of Europe. HPLC-grade acetonitrile and methanol were obtained from CHROMASOLV, while HPLC-grade formic acid was supplied by Fluka. All additional chemicals and reagents utilized in the study were of analytical grade to ensure accuracy and reliability in experimental procedures. Microcaps® disposable micropipettes, with a volume of 50 µL (catalog number: 1-000-0500), were purchased from Drummond Scientific Company, USA, to facilitate precise sample handling. Additionally, a control human plasma sample, stabilized with dipotassium ethylenediaminetetraacetic acid (K_2EDTA), was acquired from the Red Cross Society, Pakistan, for use in method validation and quality control.

Instrumentation

The quantitative analysis of the target analytes was performed using a highly sensitive LC-MS/MS system, the Exion LC™ (ABSciex, USA), which was coupled with an Applied Biosystem API4500 triple quadrupole mass spectrometer (ABSciex, Canada). This advanced mass spectrometer utilized positive electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode to enhance detection specificity and sensitivity. Several source-dependent parameters were maintained consistently for all analytes during the analysis. These included a cone gas flow rate of 50 L/h, a desolvation gas flow rate of 800 L/h, and a cone voltage of 4 kV. The source temperature was precisely regulated at 600°C to ensure optimal ionization. Additionally, the collision gas pressure was kept at 8 psi to facilitate effective fragmentation. Unit mass resolution was applied throughout the process, with the dwell time set at 100 ms to allow sufficient time for accurate data collection. All MS/MS parameters were carefully optimized through direct infusion of the analyte standards into the mass spectrometer at a flow rate of 10 µL/min. This approach ensured accurate calibration and enhanced performance of the system. Analyst software Hotfixes® version 1.6.3 (ABSciex) was employed to control and manage all LC and MS parameters, allowing for precise coordination and seamless operation during the analysis.

Liquid chromatographic and mass spectrometric conditions

Chromatographic separations were conducted using an Intersil ODS gum C18 column (4.6 × 50 mm, 3.5 µm), which was maintained at a constant temperature of 30°C to ensure stable performance during the analysis. An injection volume of 30 µL was utilized for sample introduction. The separation was achieved with a gradient mobile phase comprising methanol, 0.1% formic acid, and acetonitrile, which was delivered at a flow rate of 0.5 mL/min. The specific parameters for the gradient elution process are detailed in Table 1. This gradient approach was carefully designed to enhance the resolution of the analytes, ensuring effective separation and accurate quantification in the final chromatographic results.

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Table 1. Parameters of gradient mode

Time (min)	Mobile phase		
	0.1 % formic acid pH 3.0	Acetonitrile	Methanol
0-0.5	10	80	15
0.5-0.7	80	15	5
0.7-3.5	90	10	0
3.5-3.7	80	15	5
3.7-5.0	15	80	5

Standard solutions

Amlodipine and the internal standard (IS) were accurately weighed using an analytical microbalance and transferred into volumetric flasks. Primary stock solutions of amlodipine were prepared at a concentration of approximately 1 mg/mL, while the IS was prepared at a concentration of 0.5 mg/mL, both using methanol as the solvent. These stock solutions were stored at −20°C, where they remained stable for up to one month. To create secondary stock solutions and working solutions, the amlodipine stock solutions were diluted with methanol and water as needed. These secondary and working solutions were subsequently utilized to prepare calibration curves (CC) and quality control (QC) samples. The working stock solutions were kept at 4°C for a maximum of one week to ensure their stability and reliability during analysis. A working solution of the internal standard at a concentration of 20 ng/mL was prepared in a 50:50 (v/v) mixture of acetonitrile and methanol. Prior to spiking, blank human plasma was screened to confirm the absence of any endogenous interference at the retention times of both amlodipine and the internal standard. Calibration standard samples for amlodipine, ranging from 1 to 100 ng/mL, were prepared by spiking the blank human plasma, which had been treated with K_2EDTA, with the appropriate concentrations of amlodipine. For the assessment of precision and accuracy, control human plasma was spiked in bulk with amlodipine at designated concentrations: 3.12 ng/mL for low QC (LQC), 31.5 ng/mL for medium QC (MQC), and 81.5 ng/mL for high QC (HQC). Subsequently, 120 µL aliquots of this spiked plasma were distributed into separate tubes for analysis. All samples were stored at −80°C ± 10°C to maintain their integrity until further testing.

Sample preparation

A straightforward protein precipitation extraction method was employed to isolate amlodipine from human plasma samples. Initially, the necessary quantities of calibration curve (CC) standards and quality control (QC) samples were retrieved from the deep freezer and allowed to thaw at room temperature. For the extraction process, a 100 µL aliquot of the thawed plasma was taken, and 20 µL of the internal standard (IS) was added to this sample. To facilitate the precipitation of proteins, 300 µL of a 50:50 (v/v) mixture of acetonitrile and methanol was introduced into the mixture. This solution was then vortexed for 2 minutes to ensure thorough mixing and protein precipitation. Following the vortexing step, the sample was centrifuged at 6000 rpm for 5 minutes at 4°C to separate the solid precipitate from the liquid supernatant. After centrifugation, approximately 50 µL of the resulting supernatant was carefully transferred into appropriately labeled autosampler vials. These vials were subsequently placed in the autosampler, maintained at a temperature of 15°C ± 4°C to preserve the integrity of the samples. Finally, a 10 µL volume of each prepared sample was injected into the LC-MS/MS system for analysis, allowing for the quantification of amlodipine in the plasma samples. This extraction method is efficient and facilitates accurate detection of the analyte while minimizing potential interferences from the plasma matrix.

Method validation

A comprehensive validation of the assay was conducted in accordance with the guidelines set forth by the International Council for Harmonization (ICH) for K_2EDTA human plasma samples. This validation process encompassed all necessary parameters to ensure the reliability and accuracy of the analytical method. It included assessments of specificity, sensitivity, linearity, precision, accuracy, recovery, and stability of the analytes in the biological matrix.

The validation was meticulously designed to confirm that the assay consistently provides accurate and reproducible results, meeting the stringent requirements for bioanalytical testing. Each aspect of the validation was carefully documented, ensuring compliance with regulatory standards and enhancing the method's credibility for use in clinical and research settings.

3. Results and Discussion

In the current study, significant attention was given to optimizing and critically evaluating the composition of the mobile phase (gradient), flow rate, and choice of analytical column to achieve optimal resolution of the target peaks from endogenous components. This optimization is crucial, as it directly influences the method's reproducibility and sensitivity. The chromatographic conditions were tailored specifically for preclinical pharmacokinetic studies, ensuring that the method was both effective and efficient.

To simplify the sample preparation process and minimize solvent usage, a reduced plasma volume was employed, allowing for the use of microtubes. Initial feasibility tests were conducted with various solvent mixtures, including acetonitrile, methanol, and formic acid, alongside adjustments to the flow rates within a range of 0.1 to 0.6 mL/min. These experiments aimed to optimize chromatographic resolution for both amlodipine and the internal standard (IS).

Different analytical columns were evaluated to obtain reliable and consistent responses while maintaining a short run time. The best resolution of the peaks was achieved using an Intersil ODS gum C18 column (4.6 × 50 mm, 3.5 µm). The chromatographic separation was conducted in gradient mode using a mobile phase composed of acetonitrile, water, and formic acid. The mobile phase was delivered to the ESI chamber of the mass spectrometer at a flow rate of 0.500 mL/min, with an injection volume of 30 µL. This optimized approach facilitated the effective analysis of amlodipine, ensuring both accuracy and efficiency in the assay [21, 12-16].

The primary objective of optimizing the sample extraction process is to achieve a high extraction recovery while minimizing matrix effects, thereby enhancing the sensitivity and reliability of LC-MS/MS analysis. Ineffective extraction procedures can compromise the robustness of the method due to the presence of endogenous interferences in the sample extracts, which may not be adequately removed.

Given the advantages of time efficiency and simplicity, the protein precipitation extraction method was selected for this study. This approach allowed for effective sample preparation while ensuring that the quality of the data was maintained. The limit of quantification (LLOQ) obtained from this method was sufficient to accurately quantify amlodipine in low-dose pharmacokinetic studies.

During the analysis, amlodipine eluted at approximately 5.25 minutes. Typical chromatograms showcasing multiple reaction monitoring for amlodipine, enalapril, and the internal standard in human plasma treated with dipotassium ethylenediaminetetraacetic acid (K2EDTA) are illustrated in Fig. 2. These chromatograms provide a clear representation of the analytical performance, highlighting the distinct peaks corresponding to the analytes of interest and demonstrating the effectiveness of the chosen extraction and analytical methodologies.

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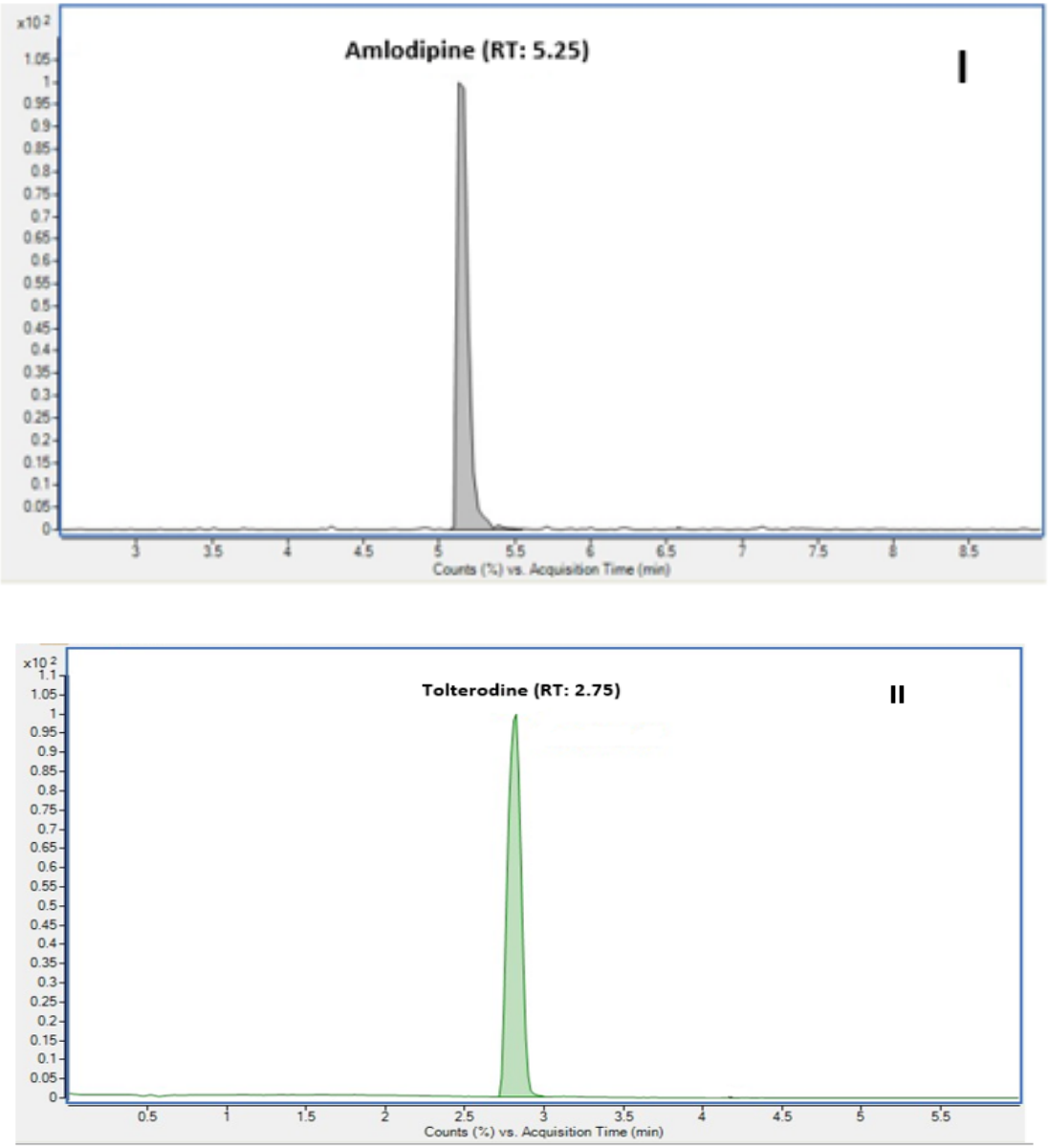

Figure 2. Typical multiple reaction monitoring chromatograms of nifedipine (I) and internal standard (II) in dipotassium ethylenediaminetetraacetic acid human blank plasma

3.1. Selectivity

The selectivity of the method was assessed using six plasma samples collected from healthy volunteers. The representative chromatograms demonstrated a clear absence of significant interference peaks in the human blank plasma spiked with amlodipine at the retention times of the analytes (as shown in Fig. 2). Specifically, the retention time for amlodipine in the spiked human plasma was found to be 5.25 minutes.

The lack of interference peaks from endogenous substances in the plasma samples indicates that the proposed method exhibits a high degree of selectivity for the target analytes in real human plasma. This selectivity is crucial for ensuring accurate and reliable quantification of amlodipine, thereby confirming the method's suitability for use in clinical and pharmacokinetic studies. The ability to distinguish the analytes of interest from potential interferences underscores the robustness of the analytical approach and enhances its applicability in routine monitoring and assessment of amlodipine therapy.

3.2. Linearity

The calibration standard curves demonstrated reliable reproducibility across the standard concentrations within the defined calibration range. The average regression value (n=3) exceeded 0.995, indicating excellent linearity. The calibration curve, which plotted the peak area ratio against concentration, exhibited a linear response for amlodipine within the working range of 1 to 130.00 ng/mL. This calibration was established using a seven-point standard curve for quantification, as illustrated in Fig. 3.

The regression equation derived from the analysis was expressed as $y=0.019x+0.1374y = 0.019x + 0.1374y=0.019x+0.1374$, where the coefficient of determination (r^2) was calculated to be 0.9952. This high r^2 value confirms the strong linear relationship between the peak area ratio and concentration of amlodipine, validating the robustness and accuracy of the assay for quantifying the drug in human plasma samples. The established calibration curve serves as a crucial foundation for the reliable quantification of amlodipine in subsequent analyses.

3.3. Recovery

The percentage mean recoveries for amlodipine at the low-quality control (LQC), medium quality control (MQC), and high-quality control (HQC) levels are detailed in Table 2. Specifically, the mean recovery rates were found to be 104.1% for LQC, 105.0% for MQC, and 108.7% for HQC.

Table 2. % Mean recovery of amlodipine for LQC, MQC and HQC

No.	LQC	MQC	HQC
1	3.14	31.2	80.0
2	3.30	29.7	72.3
3	3.05	31.1	80.1
4	3.23	30.4	88.2
5	2.95	34.8	86.0
Mean	3.13	31.4	81.3
SD	0.141	1.96	6.24
% CV	4.6	6.2	7.5
% Mean Recovery	104.2	104.0	106.7

These results indicate that the assay effectively recovers amlodipine from the plasma matrix, with recovery rates consistently above 100%. Such high recovery percentages are indicative of the method's efficiency and reliability, ensuring that it can accurately quantify amlodipine across a range of concentrations. The robust recoveries achieved at all quality control levels reinforce the method's applicability in pharmacokinetic studies and therapeutic monitoring, demonstrating its capacity to provide accurate and dependable data for clinical assessments.

3.4. Precision and accuracy

The coefficients of variation for within-run measurements ranged from 0.331% to 0.619%, indicating a high level of precision within individual runs. The within-run percentages of the nominal concentrations varied between 98.80% and 100.63%, demonstrating that the method consistently produces values close to the expected concentrations.

Similarly, the coefficients of variation for between-run measurements fell within the range of 0.332% to 0.615%, reflecting strong reproducibility across different runs. The percentages of nominal concentrations for these between-run assessments ranged from 98.98% to 101.71%, further confirming the accuracy of the assay over time.

These results are summarized in Table 3, where it is evident that the assay values for both intra-day and inter-day measurements remained well within acceptable limits. This consistent performance underscores the reliability of the method for quantifying amlodipine in human plasma, making it suitable for use in routine clinical and pharmacokinetic analyses.

Table 3. Intra-day and Inter-day precision data

Day	Intra-day precision		Inter-day precision	
	Mean	**RSD %**	**Mean**	**RSD %**
1	98.81	0.376	101.72	0.331
2	100.40	0.610	98.99	0.392
3	100.62	0.332	100.54	0.614

3.5. Matrix effect

The lowest concentration identified with a relative standard deviation (RSD) of less than 20% was designated as the lower limit of quantification (LLOQ) for amlodipine, which was established at 1.01 ng/mL. The percentage accuracy of the LLOQ samples, prepared from various biological matrix lots, was determined to be 100.5%. This value is comfortably within the acceptable range of 80.00% to 120.00% as recommended by regulatory standards for the seven different plasma lots analyzed.

Furthermore, the coefficient of variation (CV) for the LLOQ samples was recorded at 7.4%, which is well below the acceptance criterion of 20.00%. These results, detailed in Table 4, underscore the method's reliability and precision at the lower limit of quantification, reinforcing its appropriateness for accurately measuring amlodipine levels in human plasma, even at low concentrations.

Table 4. Results of matrix effect

No.	**LLQC**
1	1.02
2	0.953
3	0.903
4	1.04
5	1.07
Mean	1.04
SD	0.073
% CV	7.5
% Mean Recovery	100.7

3.6. Stability

The predicted concentrations for amlodipine at 3.12 ng/mL and 81.5 ng/mL exhibited deviations within ±15% of the fresh sample concentrations during a series of stability tests. These tests included assessments of in-injector stability over a duration of 22 hours, bench-top stability for 7 hours, and repeated stability evaluations through four freeze-thaw cycles.

Throughout these stability assessments, the results remained within the acceptable limits of assay variability, demonstrating the robustness of the method under various storage and handling conditions. This indicates that the quantification of amlodipine can be reliably performed even after prolonged exposure to different environmental factors, ensuring the integrity of the samples for accurate analysis. Such stability data is crucial for validating the method's applicability in clinical and pharmacokinetic studies, where sample integrity over time is essential for accurate drug monitoring.

4. Conclusions

In conclusion, a highly sensitive, specific, reproducible, rapid, and environmentally friendly LC-MS/MS assay for the quantification of amlodipine in human plasma, adhering to established regulatory guidelines has successfully been developed and validated. The method utilizes a straightforward precipitation technique for sample preparation, which consistently yields reliable and reproducible recovery rates.

The results obtained from this study indicate that the proposed method is not only efficient but can also be seamlessly integrated into routine analyses for amlodipine in human plasma. The validation of this method underscores its suitability for application in pharmacokinetic and bioequivalence studies, making it a valuable tool for therapeutic drug monitoring. This assay offers a reliable and practical approach for clinicians and researchers, facilitating enhanced management of amlodipine therapy and improving patient outcomes through effective drug monitoring.

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Conflicts of Interest
The authors declare no conflict of interest.

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