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Analytical Method Validation of High Performance Liquid Chromatography for Ketoconazole Tablets

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Abstract
Ketoconazole is an azole antifungal with low solubility and classified as Biopharmaceutical Classification System (BCS) Class II. A tablet formulation based on an alginate-acacia gum matrix is developed to enhance the solubility of ketoconazole. This study aims to validate the analytical method using High-Performance Liquid Chromatography (HPLC) for the assay of ketoconazole in these tablet formulations. The analysis was performed using a C18 column (250×4.6 mm, 5 µm) with a mobile phase consisting of water for injection (WFI) containing 0.15% TEA (pH 3.3) and acetonitrile (50:50 v/v), flow rate 1.0 mL/min, and detection at 232 nm. Method validation included system suitability testing, linearity, accuracy, precision, specificity, LOD, and LOQ. Specificity testing indicated no interference at the retention time of ketoconazole. Good linearity was achieved over 10–250 ppm (r = 0.9996), with good accuracy (99.8–101.75%) and precision (intraday and interday; %RSD < 2%). LOD and LOQ were 2.357 ppm and 7.142 ppm, respectively. The developed HPLC method was shown to be valid, accurate, precise, selective, and reliable for the assay of ketoconazole in tablets formulated with an alginate-acacia gum matrix. This method can be used for routine quality control.

Keywords: acacia gum; alginate; analytical validation method; HPLC; ketoconazole; tablet

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Validasi Metode Analisis Kromatografi Cair Kinerja Tinggi untuk Uji Kadar Tablet Ketokonazol

Abstrak
Ketokonazol merupakan antijamur golongan azol yangmemiliki kelarutan rendah, termasuk kedalam Biopharmaceutical Classification System (BCS) Kelas II. Formulasi tablet berbasis matriks alginat dan gum akasia dikembangkan untuk meningkatkan kelarutan ketoconazole. Penelitian ini bertujuan untuk memvalidasi metode analisis menggunakan kromatografi cair kinerja tinggi (KCKT) untuk penetapan kadar ketokonazol dalam formulasi tablet tersebut. Analisis dilakukan menggunakan kolom C18 (250×4,6 mm, 5 µm) dengan fase gerak yang terdiri dari air suntik yang mengandung 0,15% TEA (pH 3,3) dan asetonitril (50:50 v/v), laju alir 1,0 mL/menit dan deteksi pada 232 nm. Validasi metode meliputi uji kesesuaian sistem, linearitas, akurasi, presisi, spesifisitas, LOD, dan LOQ. Uji spesifisitas menunjukkan tidak ada interferensi pada waktu retensi ketokonazol. Linieritas yang baik dicapai pada rentang 10–250 ppm (r = 0,9996) dengan akurasi (99,8–101,75%) dan presisi yang baik (intraday dan interday; %RSD <2%). LOD dan LOQ masing-masing diperoleh sebesar 2,357 ppm dan 7,142 ppm. Metode HPLC yang dikembangkan terbukti valid, akurat, presisi, selektif, dan andal untuk penetapan kadar ketoconazole dalam tablet berbasis matriks alginat dan gum akasia. Metode ini dapat digunakan untuk tujuan pengendalian mutu rutin.

Kata Kunci: alginat; gum akasia; HPLC; ketokonazol; tablet; validasi metode analisis.

1. Introduction

Ketoconazole is a class of antifungal medication that acts by inhibiting adrenal cortex adrenolytic activity. The Biopharmaceutical Classification System (BCS) classifies ketoconazole as a Class II drug due to its high permeability and low aqueous solubility, and it is a weak base that dissolves only under highly acidic conditions (pH 1-3).[1,2] Various approaches have been applied to improve the solubility of ketoconazole, including the formation of co-crystals, which have the potential to achieve higher solubility than raw ketoconazole material.[3] The formation of solid dispersions, particle size reduction, and the formation of inclusion complexes with cyclodextrin have been conducted to enhance ketoconazole's solubility.[4] Research in the literature has investigated various types of alginate nanocomposites with other polymers, such as combinations of alginate with pectin, acacia gum/gum arabic, or carrageenan.[5–7]

This research used a multi-unit formulation in bead form, with the expectation that it will release the drug at a controlled rate and improve the drug's bioavailability. Tablets were chosen as the dosage form for their practicality, stability, and accurate dosing. In the assay of the active substance, High Performance Liquid Chromatography (HPLC) is a commonly used method. Tablets must meet the quality parameters stated in the compendia, including the assay that determines the content or amount of the active substance in the drug preparation. This parameter is important to ensure that each tablet contains the correct amount of active ingredient, as stated on the label, and meets the quality standards set by the compendia. Previous research has developed an analytical method for quantifying ketoconazole using HPLC as an application for supersaturation studies using ketoconazole samples encapsulated in an alginate and acacia gum matrix called beads.[8]

The assay method in this research used a reversed-phase high-performance liquid chromatography (RP-HPLC) system. The use of RP-HPLC for the analysis of basic compounds often encounters challenges, such as the formation of asymmetric chromatographic peaks and interactions between the basic compound and free silanol groups (Si-OH) on the C18 column, known as silanophilic interactions.[9,10] The free silanol groups on the silica surface have a strong tendency to interact with basic compounds, particularly through hydrogen bonding or electrostatic ion-exchange interactions, especially when the mobile phase pH is more basic.[11] Under these conditions, ionized silanol groups (SiO^-) can interact with the protonated amine groups of ketoconazole (NH_3^+), causing tailing of the chromatographic peak.[8] This problem can be minimized by adding acidifying agents such as orthophosphoric acid and HCl to the mobile phase to suppress the ionization of silanol groups and reduce unwanted interactions.[12,13] This research aims to develop an analytical method for the assay of ketoconazole tablet beads based on an alginate-acacia gum matrix using HPLC.

2. Methods

2.1. Materials

The materials used in this research included a tablet preparation based on an alginate and acacia gum composite matrix (made in the UII integrated laboratory), alginate (Shandong Jiejing Group), acacia gum (Innorex Pharma), standard reference ketoconazole (PT. Kimia Farma Mojokerto), Indonesian Pharmacopeia reference standard of ketoconazole (BPOM), distilled water, methanol (chromatography grade), acetonitrile (chromatography grade), TEA (triethanolamine), and orthophosphoric acid.

2.2. Methods

The chromatography system consisted of a Waters Alliance e2695 high-performance liquid chromatograph. The column used was a Phenomenex Luna (250×4.6 mm, 5 µm). The mobile phase was a mixture of acetonitrile and water (50:50 v/v) containing 0.15% TEA. The injection volume was 20 µL. The detection wavelength was 232 nm, and the flow rate was 1 mL/min. Samples were filtered using a 0.45 µm nylon filter before analysis. Another equipments used were analytical balance (Ohaus PR224); glasswares for preparation (Beaker glass and graduated cylinder Pyrex), glass micro-syring for beads formation, Petri dish and single punch tablet press TDP-5T (Zhengzhou Semel Machinery), hotplate stirer, ultrasonicator, micropippet, vacuum filtration, and vortex shaker.

2.3. Matrix Preparation

The polymer solution was prepared by dissolving 1 g each of sodium alginate and acacia gum in 100 mL of hot distilled water, then stirring with a magnetic stirrer until homogeneous. A 5% ketoconazole solution was prepared by dissolving 5 g of ketoconazole in distilled water to a final volume of 100 mL.

The polymer mixture was then prepared with an alginate:acacia ratio (75:25) by mixing 15 g of alginate solution and 5 g of acacia gum solution, then adding 10 mL of the ketoconazole solution and stirring until uniform. This mixture was dripped into a solution of 3.75 g $CaCl_2$ in distilled water to form beads. The formed beads were then dried in an oven at 50°C for 24 hours.

2.4. Tablet Formulation

Tablet components were weighed according to the predetermined formulation. The tablet formulation included 500 mg beads (equivalent to the normal dose ketoconazole of 200 mg from the data of the previous study)[2], 163.75 mg of Avicel 102 as a filler/diluent, 75 mg of starch as a disintegrant, 7.5 mg of magnesium stearate as a lubricant, and 3.75 mg of Aerosil as a glidant. After weighing, the active ingredient (beads) was mixed with the excipients. The active ingredient was mixed with Avicel 102 and starch for 20 minutes. Subsequently, magnesium stearate and Aerosil were added, and the mixture was homogenized for an additional 2 minutes. The uniformly mixed powder blend was then compressed into tablet form using a single-punch tablet press. The preparation of the tablet was adopted from the previous research.[14]

2.5. Mobile Phase Preparation

The mobile phase was prepared by mixing Water for Injection (WFI) with 0.15% TEA (750 µL) and acetonitrile (50:50). The pH was adjusted to 3.3 with orthophosphoric acid solution.

2.6. Standard Solution Preparation

A stock solution was prepared by dissolving 50 mg of ketoconazole in 100 mL of methanol to obtain a final concentration of 500 ppm. Subsequently, the solution was sonicated to dissolve the compound and remove any remaining air fully. A standard calibration curve was constructed from the stock solution, diluted with mobile phase solution, to obtain final concentrations ranging from 10 to 250 ppm.

2.7. System Suitability testing

A 50 ppm standard solution was injected six times, and the relative standard deviation (RSD) was determined. The system suitability test was considered acceptable if the RSD value for the peak area was ≤ 2.0%, the difference in retention time (tR) was ≤ 1.0%, the resolution (Rs) was ≥ 2, and the number of theoretical plates (N) was > 2000.

2.8. Specificity evaluation

Analysis was performed using the mobile phase, methanol, HCl, and a blank polymer combination bead mixture without ketoconazole. The results were considered satisfactory for each peak and their proximity to one another if the Rs value was ≥ 2. All of the validation parameters evaluations were adopted from the ICH guideline for analytical method validation.[15]

2.9. Linearity evaluation

A concentration series of 10, 50, 100, 150, 200, and 250 ppm was prepared from a 1000 ppm standard stock solution and diluted with the mobile phase. The calibration curve was obtained from the concentration and the area under the curve (AUC). Linearity was considered good if the correlation coefficient (r) was ≥ 0.999 from the linear regression equation.

2.10. Precision and Accuracy test

Precision testing was conducted both intraday and interday by measuring standard solutions at concentrations of 50, 100, and 150 ppm. Intraday precision measurements were performed three times for each concentration on the same day, while interday measurements were conducted over three consecutive days for each concentration. Precision was considered acceptable if the %RSD was ≤ 2.0%. Accuracy testing was performed using the standard addition method. A 500 µL volume of standard solution was added at concentrations of 50%, 100%, and 150% of the sample concentration of ketoconazole in the preparation (1:1). Each concentration series was replicated three times. Accuracy was considered to meet the criteria if the percentage recovery was between 98.0% and 102.0%.

2.11. Limit test

The limit of detection (LoD) and limit of quantity (LoQ) testing were carried out by preparing a concentration series of 1, 2, 4, 6, and 8 ppm from a 100 ppm standard stock solution, which was then diluted in a 5 mL volumetric flask using the mobile phase. The LoD is the smallest amount of analyte in a sample that can be detected, while the LoQ is the lowest amount of analyte that can be quantified with acceptable precision.

The data from the sample measurements using HPLC were processed in Microsoft Excel to calculate the average sample area, linear regression equation, correlation coefficient, standard deviation, coefficient of variation, calibration curve, % recovery, and standard deviation (SD). Conclusions were then drawn by comparing the test results with reference values.

3. Result

The analytical method used was Reversed-phase High-Performance Liquid Chromatography (RP-HPLC). The chromatographic system employed a C18 column measuring 250×4.6 mm with 5 µm particles. The mobile phase consisted of a mixture of WFI, 0.15% TEA, and acetonitrile (50:50), adjusted to pH

3.3 with orthophosphoric acid. This pH was selected because ketoconazole is weakly basic, to keep it in its ionized form and enhance its solubility. Ketoconazole is a weakly basic drug with pKa values of 2.94 and 6.51, respectively.[16] Detection of the compound was carried out at 232 nm, the maximum wavelength (λ max) of ketoconazole, which provides more optimal readouts.[17]

3.1. System suitability test

The HPLC system was optimized to demonstrate system suitability (Table 1), including retention time (to ensure system stability), peak area and %RSD (to determine reproducibility), asymmetry factor (to evaluate peak shape), and number of theoretical plates (to measure column efficiency). System suitability was assessed by 6 replicate analyses of ketoconazole bead tablets at 100 ppm.

3.2. Specificity evaluation

The specificity of the analytical method was rigorously evaluated to ensure that the measured signal was solely attributable to ketoconazole, without interference from the formulation's excipients or other components. This validation parameter is critical to ensuring the assay's accuracy and reliability, as false positives or inflated results could lead to incorrect potency assessments. Specificity was demonstrated by comparing the chromatograms of a placebo formulation (containing all inert components), the active ketoconazole standard, and the finished sample. The analysis confirmed that no interfering peaks were detected at the retention time of ketoconazole (3.766 minutes), thereby confirming the method's selectivity for the target analyte. The chromatogram justifying this statement is provided in Figure 1. As matrix components, sodium alginate and acacia gum absorbed at different wavelengths from ketoconazole. Sodium alginate absorbed at 276.7 nm18 while the acacia gum absorbs at around 300 nm, and band formation occurs between 350 and 375 nm.[19]

3.3. Linearity test and range

The ability to produce results that are directly proportional to the analyte concentration in the sample is how ICH Q2 defines linearity. A plot of concentration series, with a minimum of six series concentrations, should be performed in triplicate to determine linearity.[20] According to EMA, the calibration curve's outcome should be proportionate to both concentration and area under peak21 The coefficient (r^2), slope (b), and intercept (a) were used to evaluate linearity. The capacity of the method to provide a proportional correlation between the concentration and the sample is known as linearity. The calibration curves showed a linear relationship with sample concentration

A calibration curve was constructed using methanol as the solvent for standard solutions at concentrations of 10, 50, 100, 150, 200, and 250 ppm. The concentration range of 10-250 ppm was selected based on the expected ketoconazole concentration in the tablets (typically 200 mg/tablet). Based on the calibration curve results, the regression equation obtained was y = 41481x – 62738 (Figure 2). The correlation coefficient is 0.9996, indicating a strong linear relationship between peak area and concentration.

3.4. Precision and accuracy evaluation

The accuracy test in this study used the standard addition method because it is more applicable to complex matrices such as bead tablets. The degree to which the test result agrees with the true value or a recognized reference is known as the analytical method's trueness.[22] The percentage recovery in this approach at three different concentration levels (80%, 100%, and 120%) with three replicates from each concentration was assessed using the usual addition method. The percentage recovery in this study was determined using the standard addition method at 3 different concentration levels. The obtained percentage recovery data ranged from 99.8% to 101.75% as compiled in Table 2. The results of this accuracy test meet the requirements based on AOAC requirements, namely 90-107% (for sample concentrations of ~100 ppm).[23]

3.5. Limit of detection (LoD) and limit of quantification (LoQ)

To ascertain the devised method's sensitivity, the LoD

Table 1. The results from the system suitability test

Parameter	Acceptance criteria	Result
Retention time (minutes)	RSD <2	3.561 ± 001(n=6) RSD: 0.45%
Precision of the area under the curve	RSD <2	7959659 ± 8237(n=6) RSD: 0.1%
Asymmetry factor	< 2	1.4 ± 0.01(n=6)
Theoretical plate number (N)	> 2000	4130 ± 73(n=6)

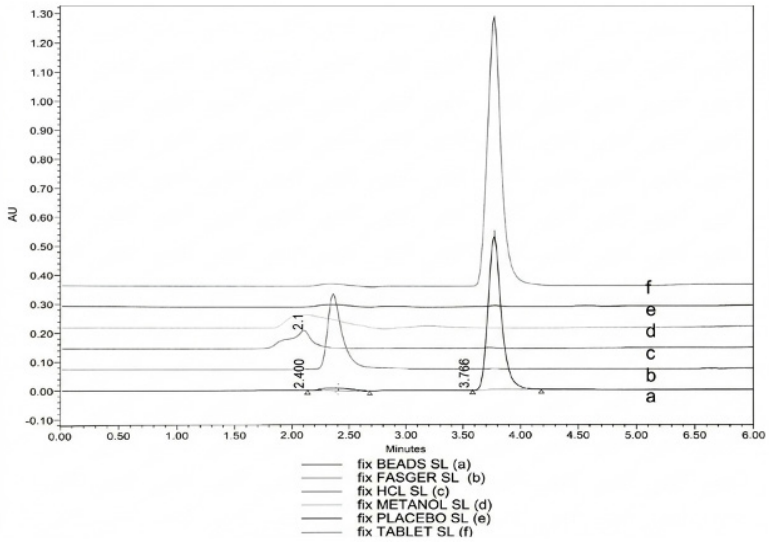

Figure 1. Chromatograms of beads without excipients (a), mobile phase (b), HCl (c), methanol (d), placebo (e) and sample tablet (f)

and LoQ were calculated. Although it is not crucial to determine, the LoD denotes the lowest concentration of the analyte in a sample. In chromatography, the injected sample that yields a peak height that is at least two or three times higher than the baseline noise level is known as the detection limit. The least injected quantity that yields adequate accuracy and precision measurement is known as the LoQ. If the peak heights are 10–20 times the baseline noise level, the precision will yield an RSD of less than 10–15%.[24,25] For ketoconazole, the LOD result was 2.357 ppm, which was detectable with reliability. The ketoconazole LoQ value was 7.142 ppm, representing the lowest concentration of the analyte that can be quantified with acceptable accuracy and precision.

3.6. Assay of the tablet

The determination of ketoconazole content in the bead tablets was evaluated using the mobile phase after dissolution in 100 mL of methanol. The average result was 183.07 ± 1.72 mg, with a %RSD of 0.95.

4. Discussion

A study conducted by Olga et al. regarding the validation of ketoconazole pharmaceutical dosage forms using HPLC instruments reported recovery values ranging from 92.67% to 105.23%.[26] In comparison, the current study achieved superior recovery values between 99.8% and 101.75%, suggesting that the HPLC method employed here offers higher accuracy than conventional HPLC methods. Several previous studies have validated methods for measuring ketoconazole dissolution concentrations, typically assessing linearity, precision, accuracy, specificity, and robustness for standard tablet formulations. Those studies reported ketoconazole concentrations

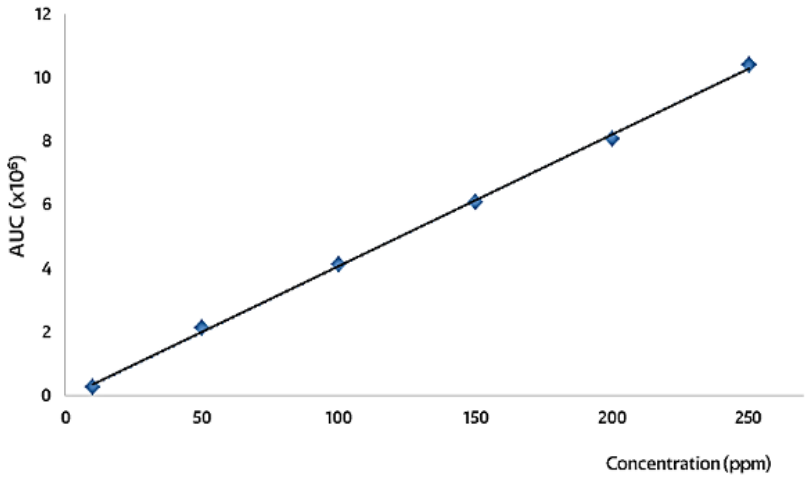

Figure 2. Calibration curve employed in linearity evaluation

Table 2. Result of accuracy and intraday precision evaluation using 3 levels of concentration with standard addition

Conc.level (%)	Conc of standard (ppm) ± SD	Sample conc (ppm) ± SD	Total conc (ppm) ± SD	%Recovery
80	55.66±0.08	191.42±0.47	247.00±0.09	99.84
100	106.91±0.05	191.42±0.47	300,20±0.45	101.75
120	158.22±0.36	191.42±0.47	352.00±0.23	101.49

ranging from 90.0% to 110.0%.[27] Furthermore, while previous research primarily focused on validating ketoconazole in conventional tablet matrices and evaluating parameters such as linearity, precision, and robustness, this study addresses the complexity of ketoconazole-loaded beads formulated into tablets. Standard tablet validations typically report concentrations within the 90.0%-110.0% range. Our findings indicated that the 500 mg bead tablets, containing 200 mg of ketoconazole, yielded an average content of 183.07 ± 1.72 mg (91.5%). Although the bead matrix provides a more complex delivery system than conventional tablets, the results remain well within the official requirements of the Indonesian Pharmacopoeia (90–110%), confirming that the formulation and the analytical method are both robust and compliant with pharmaceutical standards.[28,29]

The chromatographic conditions optimized in this study are consistent with previous research on ketoconazole analysis, particularly regarding the use of a reversed-phase system. Previous studies utilizing the shake flask solubility method to determine ketoconazole solubility in phosphate buffer (pH 6.8) employed a similar mobile phase composition that reported high precision and accuracy, with a correlation coefficient (r^2) of 0.9997 and recovery values between 99.556% and 101.216% within a low concentration range of 0.5–12 ppm.[29]

In comparison, while the fundamental mobile-phase and detection parameters remain aligned, our study demonstrates the robustness of this HPLC method when scaled to a significantly wider concentration range (10–250 ppm). This higher range is essential for the direct quantification of ketoconazole in bead-loaded tablets (200 mg/tablet). In contrast, the previous study focused on lower concentrations typical of solubility testing.[30] Despite the increase in concentration and the complexity of the bead matrix, our method maintained excellent linearity (r^2 =0.9996) and high accuracy, with recovery values (99.8% to 101.75%) that are closely comparable to the results obtained in the solubility study. Furthermore, our findings confirm that the inclusion of polymer matrices, such as sodium alginate and acacia gum, does not compromise the method's selectivity, as previously validated in simpler buffer systems. This comparison underscores that the developed method is not only highly accurate for specialized solubility studies but also versatile enough for the quality control of complex, solid dosage forms such as polymer-based beads.

The robustness of the developed HPLC method is further evidenced when compared to specialized analytical protocols, such as the two-stage biorelevant dissolution model. Previous research using a pH-shift approach to simulate the gastrointestinal transition from simulated gastric fluid (SGF) to fasted-state simulated intestinal fluid (FaSSIF) employed a similar reversed-phase C18 system with detection at 232 nm. That study reported a retention time of approximately 3.5 minutes and high accuracy, with recoveries ranging from 100% to 103%.[31]

While the chromatographic parameters (column type, wavelength, and flow rate) are remarkably consistent with our findings, there are distinct differences in the

Table 3. Interday precision evaluation results

Conc. level (%)	Day 1	Day 2	Day 3	Mean± SD (n=27)	%RSD
	119.24	120.42	116.56		
50	119.75	119.64	117.61	118.83±1.35	1.14
	119.67	119.19	117.66		
	152.99	151.64	151.55		
100	152.44	151.60	151.53	151.95±0.60	0.40
	152.53	151.52	151.80		
	176.23	178.55	182.42		
150	176.02	179.05	182.56	179.21±3.27	1.83
	175.76	179.70	182.65		

applications and matrix complexities. The biorelevant study focused on tracking supersaturation profiles over time in liquid media to mimic physiological pH changes. In contrast, our study validates the method for quantifying ketoconazole in a solid dosage form (bead-loaded tablets). Despite the absence of complex polymers such as sodium alginate and acacia gum in standard biorelevant media, our method achieved a more precise recovery range of 99.8% to 101.75% and comparable linearity (r^2 = 0.9996 vs 0.9995). This comparison highlights that the proposed HPLC method is not only sensitive enough for kinetic studies in complex biorelevant fluids but also sufficiently robust to overcome potential matrix interferences from novel polymer-based delivery systems. Consequently, the method demonstrates high versatility, serving as a reliable tool for both advanced in vitro dissolution modeling and routine quality control of innovative ketoconazole formulations.

5. Conclusion

This study successfully developed and validated an analytical method using HPLC for the determination of ketoconazole in tablets formulated with an alginate-acacia gum matrix. The selection of this natural matrix aimed to enhance the solubility and bioavailability of ketoconazole, a BCS Class II drug. The HPLC method used demonstrated validity based on system suitability tests, linearity (r = 0.9996), accuracy (% recovery, 99.8–101.75%), precision (%RSD <2%), and specificity. Therefore, this method has been proven to be selective, accurate, precise, and applicable to the routine assay and quality control of ketoconazole tablet preparations using composite beads in the pharmaceutical industry.

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Conflict of Interest

The authors declare no conflicts of interest.

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