STABILITY-INDICATING HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF FORMOTEROL FUMARATE DIHYDRATE AND FLUTICASONE PROPIONATE IN BULK DRUG AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: The objective of the present work was to develop validated stability-indicating high-performance thin-layer chromatographic method for simultaneous estimation of formoterol fumarate dihydrate (FFD) and fluticasone propionate (FP) in bulk drug and pharmaceutical dosage form.

Methods: Pre-coated silica gel aluminum plates 60 F-254 were used as stationary phase. The mixture of toluene:ethyl acetate:formic acid (98%) (6:4:0.1; v/v/v) was used as a mobile phase. The densitometric quantification was carried out at 233 nm. The method was validated according to the ICH guidelines. The specificity and stability indicating the capability of the method were proven through degradation studies. Both drugs were subjected to acid (0.1N HCl) and base (0.1N NaOH) hydrolysis, oxidation (3% v/v H₂O₂), photolytic, and neutral degradation conditions.

Results: The selected mobile phase resolved peaks of FFD and FP with R values 0.27±0.10 and 0.64±0.10, respectively. Determination coefficients of calibration curves were found to be 0.998 and 0.999 in the range of 1–3.5 µg/spot and 10–60 µg/spot for FFD and FP with an accuracy of 99.09% for FFD and 99.20% for FP. The degradation products of FFD and FP were resolved from the pure drug with significant differences in their retention factor values.

Conclusion: The developed method is simple, accurate and can be successfully applied for quantification of FFD and FP in bulk drug and pharmaceutical dosage form, contributing to improve the quality control and assure the therapeutic efficacy.

Keywords: Formoterol fumarate dihydrate, Fluticasone propionate, High-performance thin-layer chromatography, Stability-indicating method, Validation.

INTRODUCTION

Inhalation is currently the preferred route of drug delivery in asthma in accordance with a global initiative for asthma guideline [1], as it allows the release of drug directly to the site where the action is needed, thus minimizing systemic side effect. Inhaled corticosteroid in combination with a long-acting β2-agonist is the gold standard for the management of persistent asthma, with maximal targeting and minimal systemic side effects. Formoterol fumarate dihydrate (FFD) is a long-acting selective β-2 agonist used as a bronchodilator in the treatment of asthma. Chemically, FFD is a (E)-but-2-enedioic acid; N-[2-hydroxy-5-[(1S)-1-hydroxy-2-[[2S)-1-(4-methoxyphenyl) propan-2-yl] amino] ethyl] phenyl[formamide [2,3]. Fluticasone propionate (FP) is chemically 6α, 9-Difluoro-17-[[fluoromethyl] sulphonyl] carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrost-1-4-dien-17α-y1propanoate. FP is a tri-fluorinated glucocorticoid specifically designed to provide enhanced anti-inflammatory effect [2,4]. Both drugs are official in IP, BP, EP, and USP [5-8]. The chemical structures of FFD and FP [8] are shown in Fig. 1a and b, respectively.

Literature survey for FFD and FP revealed that various analytical methods using techniques such as high-performance liquid chromatography [9-20], spectrophotometry [18,21-25], and high-performance thin-layer chromatography (HPTLC) [19,26] were reported for quantitative determination of single or multi-component systems. Gowekar and Wadher reported HPTLC method for simultaneous estimation of FFD and FP, but no degradation profile has been stated in the literature [27]. To the best of our knowledge, there is no stability indicating HPTLC method reported for the simultaneous estimation of FFD and FP in bulk drug and pharmaceutical dosage form.

Hence, the objective of the present work was to develop and validate the stability indicating HPTLC method for simultaneous estimation of FFD and FP in bulk drug and pharmaceutical dosage form.

METHODS

Chemicals and reagents
Gift samples of FFD and FP were procured from Yansi Laboratories Pvt. Ltd. Solapur, Maharashtra, India. The pharmaceutical formulation of capsule Maxiflo® Rotacaps containing 6 µg of FFD and 100 µg of FP manufactured by Cipla Ltd. was procured from the market. All analytical grade chemicals and reagents used for the analysis were purchased from Merck, Mumbai, India.

Instrumentation
Pre-coated silica gel aluminum plates 60F-254 (20 cm×10 cm, 250 µm thickness, E. Merck, Darmstadt, Germany) supplied by Anchorn, Mumbai were used. The sampling was done by automated TLC sampler Linomat V applicator (Camag, Muttenz, Switzerland) which was controlled by Win-Cats software (V 3.15, Camag, Muttenz, Switzerland). The standard and sample solutions were spotted in the form of bands of width 6 mm with a Camag 100 µL sample (Hamilton, Bonaduz, Switzerland) syringe. Linear ascending development was carried out in a twin trough glass chamber (20 cm×10 cm, 10 cm×10 cm Camag, Muttenz, Switzerland). The mobile phase consisted of toluene:ethyl acetate:formic acid (98%)
Pharate and Dhaneshwar

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The developing solvent was run up to 80 mm and development was performed at room temperature (25°C±2°C) at a relative humidity of 60%±5%. The development time was 20 min. Plates were scanned at 233 nm with CAMAG TLC scanner 3. Deuterium lamp emitting a continuous UV spectrum between 200 and 400 nm was used as a source of radiation.

**HPTLC method and chromatographic conditions**

**Preparation of standard stock solutions**

Accurately weighed 10 mg of FFD was transferred to 10 ml volumetric flask, dissolved and diluted up to the mark with methanol (1 mg/ml).

Accurately weighed 10 mg of FP was transferred to 10 ml volumetric flask, dissolved and diluted up to the mark with chloroform (1 mg/ml).

**Preparation of sample solution**

Powder from 20 capsules (Maxiflo-100 Rotacaps containing 6 μg of FFD and 100 μg of FP per capsule) were weighed, their average weight determined (3.038 mg) and crushed to fine powder. The quantity of powder equivalent to 10 mg of FP and 0.6 mg of FFD was transferred into a 10 ml volumetric flask containing 5 ml of methanol and mixed well. The solution was ultrasonicated for 20 min, and then diluted to 10 ml with methanol. The solution was filtered through Whatman filter paper (0.45 μm). The amount of each drug present in the sample was determined by comparing mean peak areas with that of the standard.

**Prewashing of plates**

Densitometric estimation was carried out on 20 cm×10 cm pre-coated silica gel 60F–254 plates from E. Merck. The plates were pre-washed with methanol, dried and activated for 15 min at 110°C before chromatography.

**Selection of the solvent**

Methanol and chloroform were selected as solvents for preparing sample solutions.

**Sample application**

Powder from 20 capsules (Maxiflo-100 Rotacaps containing 6 μg of FFD and 100 μg of FP per capsule) were weighed, their average weight determined (3.038 mg) and crushed to fine powder. The quantity of powder equivalent to 10 mg of FP and 0.6 mg of FFD was transferred into a 10 ml volumetric flask containing 5 ml of methanol and mixed well. The solution was ultrasonicated for 20 min, and then diluted to 10 ml with methanol. The solution was filtered through Whatman filter paper (0.45 μm). The amount of each drug present in the sample was determined by comparing mean peak areas with that of the standard.

**Optimization of the mobile phase**

Various solvent systems such as mixtures of (1) n-hexane:ethyl acetate:methanol:acetic acid (2.0:2.5:2.0:0.2; v/v/v/v), (2) n-hexane:ethyl acetate:methanol:formic acid (2.0:2.5:2.0:0.2; v/v/v/v), (3) n-hexane:ethyl acetate:acetic acid (5:10:0.2; v/v/v), and (4) toluene:ethyl acetate:formic acid (7:3:0.1; v/v/v) were tried to separate and resolve spots of FFD and FP from each other and other excipients of formulation. The mixture of n-hexane:ethyl acetate:methanol:acetic acid (2.0:2.5:2.0:0.2; v/v/v/v) and n-hexane:ethyl acetate:methanol:formic acid (2.0:2.5:2.0:0.2; v/v/v/v) provided well-resolved peaks but tailing was observed. Good peak shape was observed with a mixture of toluene:ethyl acetate:formic acid (7:3:0.1; v/v/v), but the FFD did not resolve from FP. Finally, the mixture of toluene:ethyl acetate:formic acid (6:4:0.1; v/v/v) showed well-resolved peaks with better peak shape. FFD and FP were satisfactorily resolved with Rf value 0.27±0.10 and 0.64±0.10, respectively. Pre-saturation of TLC chamber with the mobile phase for 20 min assured better reproducibility in the migration of FFD and FP with better resolution which is shown in Fig. 3.

**Method validation**

The developed HPTLC method was validated as per the ICH guidelines Q1A (R2), Q1B, Q2 (R1) for linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and specificity [28-33].

**Linearity**

The linearity of the method was evaluated by constructing calibration curves at six concentration levels. Aliquots of standard working solution of FFD (1, 1.5, 2, 2.5, 3, and 3.5 μL) and (10, 20, 30, 40, 50, and 60 μL) of FP were applied on the plate, to obtain concentrations of 1, 1.5, 2, 2.5, 3, and 3.5 μg/spot for FFD and 10, 20, 30, 40, 50, and 60 μg/spot for FP. The calibration curves were constructed by plotting peak area versus concentration with the help of Win-CATS software. The plate was developed in a twin trough glass chamber, using 20 min chamber saturation time. The length of the run was 80 mm. The developed plates were air-dried. Scanning was performed in UV mode at 233 nm. The slit dimension was kept at 5×0.45 mm at a scanning speed of 100 nm/s.

![Fig. 1: (a) Chemical structure of formoterol fumarate dihydrate. (b) Chemical structure of fluticasone propionate](image-url)

![Fig. 2: Overlay spectra for selection of detection wavelength (233 nm)](image-url)
Slope, intercept, and coefficient of determination ($r^2$) of the calibration curves were calculated to ascertain linearity of the method.

**Precision**

To evaluate intraday precision, three samples at three different concentrations were analyzed on the same day. The interday precision was studied by comparing assays performed on three different days.

The precision of an analytical method expresses the degree of scatter between a series of measurements obtained from multiple samples of the same homogeneous sample under prescribed conditions.

The intraday and interday variation for determination of FFD and FP were carried out at three different concentration levels 1.5, 2, and 2.5 µg/spot for FFD and 20, 30, and 40 µg/spot for FP.

**Repeatability**

Repeatability of sample application was assessed by spotting 2 µg/spot for FFD and 30 µg/spot FP of standard drug solution 6 times on a TLC plate at different times on the same day by sample applicator, followed by the development of plate and recording of the peak areas for six spots.

**Accuracy**

Accuracy studies were carried out at 80–120% levels, by mixing a known quantity of standard drug (0.5, 0.6, and 0.7 µg for FFD and 8, 10, and 12 µg for FP) with the sample formulation and the contents were analyzed by the proposed method.

**Specificity**

The specificity of the method was ascertained by analysis of drug standards and samples. The identities of the peaks for FFD and FP were confirmed by comparing the $R_f$ with those of standards. The peak purity of FFD and FP was assessed by comparing their respective spectra at peak start, peak apex, and peak end positions of the spot.

**Robustness**

The proposed HPTLC method was tested for robustness. The parameters selected for the robustness study were, change in the amount of toluene in mobile phase composition, change in time from spotting to chromatography and time from chromatography to scanning, and change in saturation time. By introducing small changes in these parameters, the effect on the results was examined.

**Limit of detection (LOD) and limit of quantitation (LOQ)**

LOD and LOQ values represent the sensitivity of the proposed analytical method. To estimate the LOD and LOQ, blank methanol was spotted 6 times. Different concentrations 0.1, 0.3, 0.5, 0.8, and 1 µg/spot for FFD were spotted 6 times.
The neutral degradation of FFD and FP in combination was induced by refluxing them together with 10 ml of water at 50°C for 2 h. Samples were withdrawn (0.5 ml) at different time intervals for 2 h. 3 µL solution was applied on TLC plate in such a way that final concentration achieved was 1.8 µg/spot for FFD and 30 µg/spot for FP and densitograms were developed.

RESULTS

Optimization of chromatographic conditions
Toluene:ethyl acetate:formic acid (98%): (6:4:0.1 v/v/v) mixture provided best resolution with better peak shape. The R_f values were found to be 0.27 and 0.64 for FFD and FP, respectively.

### Table 1: Linearity and range for FFD and FP

| Linearity and range | FFD | FP |
|---------------------|-----|----|
| Range (µg/spot)     | 1–3.5 | 10–60 |
| Regression coefficient (r^2) | 0.998 | 0.999 |
| Linearity equation | y=677.6x+472.73 | y=1013.3x+10059 |

FFD: Formoterol fumarate dihydrate, FP: Fluticasone propionate

### Table 2: Precision studies for FFP and FP

| Drug | Precision | Concentration (µg/spot) | Area | Average area | Standard deviation | % RSD |
|------|-----------|-------------------------|------|--------------|-------------------|-------|
| FFD  | Intraday  | 1.5                     | 1476 | 1452         | 1448              | 1460  | 15.14 | 0.03 |
|      | 2         | 1791                    | 1798 | 1757         | 1782              | 1782  | 21.93 | 1.23 |
|      | 2.5       | 2234                    | 2204 | 2252         | 2230              | 2230  | 24.24 | 1.08 |
|      | 1.5       | 1481                    | 1462 | 1498         | 1480              | 1480  | 18.00 | 1.21 |
|      | 2         | 1771                    | 1742 | 1789         | 1767              | 1767  | 23.71 | 1.34 |
|      | 2.5       | 2267                    | 2244 | 2212         | 2241              | 2241  | 27.62 | 1.23 |
|      | Interday  | 1                      | 38152| 38869        | 37878             | 38299 | 511.7 | 1.33 |
|      | 30        | 49462                   | 49758| 48935        | 49551             | 49551 | 543.7 | 1.09 |
|      | 40        | 57938                   | 57939| 56901        | 57630             | 57630 | 631.9 | 1.09 |
|      | 1         | 38282                   | 37095| 37248        | 37808             | 37808 | 522.4 | 1.38 |
|      | 30        | 47989                   | 48102| 48966        | 48322             | 48322 | 566.9 | 1.17 |
|      | 40        | 57767                   | 56894| 56587        | 57082             | 57082 | 612.2 | 1.07 |
| FP   | Intraday  | 20                      | 38512| 38989        | 38437             | 38699 | 511.7 | 1.33 |
|      | 30        | 49562                   | 49758| 48935        | 49551             | 49551 | 543.7 | 1.09 |
|      | 40        | 57938                   | 57939| 56901        | 57630             | 57630 | 631.9 | 1.09 |
|      | 1         | 38282                   | 37095| 37248        | 37808             | 37808 | 522.4 | 1.38 |
|      | 30        | 47989                   | 48102| 48966        | 48322             | 48322 | 566.9 | 1.17 |
|      | 40        | 57767                   | 56894| 56587        | 57082             | 57082 | 612.2 | 1.07 |

FFD: Formoterol fumarate dihydrate, FP: Fluticasone propionate, RSD: Relative standard deviation
Validation of the method

Linearity (calibration curve)

Linearity was demonstrated with six different concentration levels for both FFD and FP, which were found to be linear in the range of 1–3.5 μg/spot for FFD and 10–60 μg/spot for FP. The values are given in Table 1. Regression coefficient and concentration of the drugs correlated well. The calibration curves are shown in Figs. 4 and 5. The residual plots are shown in Figs. 6 and 7.

Precision

The values of intraday and interday precision are given against sample application and scanning of peak area and results are expressed in terms of percentage relative standard deviation (RSD). The measurement of peak areas at three different concentration levels showed a low value of percentage RSD (<2) for intra- and inter-day variation, which suggested that the method was precise (Table 2).

Repeatability

The percentage RSD for repeatability of the drugs was found to be <2 (i.e., 1.05 for FFD and 1.17 for FP). Hence, it was concluded that the proposed method for estimation of FFD and FP was repeatable in nature; the data for the same are shown in Table 3.

| Drug                          | Concentration (µg/spot) | Peak area | Average area | % RSD |
|-------------------------------|-------------------------|-----------|--------------|-------|
| Formoterol fumarate dihydrate | 2                       | 1763      | 1773.33      | 1.05  |
| Fluticasone propionate        | 30                      | 48369     | 48707.16     | 1.17  |

FFD: Formoterol fumarate dihydrate, FP: Fluticasone propionate, RSD: Relative standard deviation

Accuracy

To check the accuracy of the method, recovery studies were carried out by standard addition of drug solution to pre-analyzed sample solution at

Table 4: Recovery studies for FFD and FP by HPTLC method (n=3)

| Label claim (µg/capsule) | % Level of spiked standard drug | Conc. added | Formulation | Pure drug | Total amount (µg) | Amount recovered (µg) | % Recovery Mean (% recovery) |
|--------------------------|---------------------------------|-------------|-------------|-----------|------------------|------------------------|-----------------------------|
| FFD 6 µg                 |                                  | 80          | 0.6         | 0.5       | 1.1              | 1212                   | 99.09 99.13                 |
|                          |                                  | 100         | 0.6         | 0.6       | 1.2              | 1279                   | 99.08 99.12                 |
|                          |                                  | 120         | 0.6         | 0.7       | 1.3              | 1348                   | 99.23 99.23                 |
| FP 100 µg                |                                  | 80          | 10          | 8         | 18               | 28136                  | 99.05 99.22                 |
|                          |                                  | 100         | 10          | 10        | 20               | 30195                  | 99.35 99.24                 |
|                          |                                  | 120         | 10          | 12        | 22               | 32196                  | 99.27 99.27                 |

FFD: Formoterol fumarate dihydrate, FP: Fluticasone propionate, RSD: Relative standard deviation, HPTLC: High-performance thin-layer chromatography

Table 5: Results of robustness evaluation of FFD and FP (n=3)

| Condition                              | FFD | | RSD | | RSD |
|----------------------------------------|-----|---|-----|---|-----|
| A: Change in amount of toluene in mobile phase composition | 1413 | 1.13 | 57047 | 1.14 |
| B: Change in saturation time (min)     | 1471 | 1.12 | 57006 | 1.12 |
| C: Time from spotting to chromatography (+10 min) | 1433 | 1.10 | 56953 | 1.13 |
| D: Time from chromatography to scanning (+10 min) | 1464 | 1.14 | 57118 | 1.07 |

FFD: Formoterol fumarate dihydrate, FP: Fluticasone propionate, RSD: Relative standard deviation
three different levels 80, 100, and 120%. The percent mean recoveries were found to be 99.13% for FFD and 99.22% for FP (Table 4).

### Specificity

The peak purity of FFD and FP was assessed by comparing their respective densitograms at peak start, peak apex, and peak end positions of the spot, i.e., r (start, middle) = (0.25–0.27) and r (middle, end) = (0.27–0.29) for FFD and r (start, middle) = (0.57–0.65) and r (middle, end) = (0.65–0.68) for FP. The chromatogram of a capsule sample showed peaks at Rf values of 0.27 and 0.64 for FFD and FP, respectively (Fig. 8), indicating that there is no interference of the excipients present in the capsule formulation indicating the specificity of the method.

### Robustness

The percentage RSD of the peak areas was calculated for change in amount of toluene in mobile phase composition, change in time from spotting to chromatography and time from chromatography to scanning, change in saturation time and change in solvent run distance (Table 5).

### Limits of detection and quantitation

The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3:1). The LOD was found to be 0.3 µg/spot for FFD and 0.2 µg/spot for FP. LOQ is the smallest concentration of the analyte, which gives the response that can be accurately quantified (signal to noise ratio of 10:1). The LOQ was 1 µg/spot for FFD and 0.6 µg/spot for FP which indicates that the proposed method was sensitive enough to detect the drugs at very low concentration level (Table 6).

### Forced degradation studies

Selectivity of the method was demonstrated by enhancing degradation of FFD and FP under various stressed conditions (acid, base hydrolysis, oxidation, neutral, and photochemical), to show that FFD and FP were separated from their possible degradation products. The number of degradation products with their Rf was calculated and listed in Table 7.

### Acid-induced degradation

The densitograms for acid degraded FFD and FP showed additional peaks at Rf, 0.15, 0.47 and 0.72, respectively. The rate of degradation for FFD (18.00%) was more as compared to FP (13.22%) in acid-induced degradation (Fig. 9).

### Base-induced degradation

The degradation in 10 ml of 0.1 N NaOH at 50°C was so fast that around 18.12% of FP was degraded in 15 min, forming one degradation product at Rf, 0.78 and Rf 0.11% of FFD was degraded with one degradation product at Rf, value 0.42 (Fig. 10).

### Oxidative degradation

The drugs were found to be susceptible to oxidative degradation. The densitogram of hydrogen peroxide-induced degradation showed the additional peaks at Rf value 0.50 for FFD and 0.72 and 0.84 for FP, respectively. The percent of degradation was found to be 13.06% for FFD and 11.29% for FP (Fig. 11).

### Photolytic degradation

FP and FFD were found to undergo photolytic degradation after exposure of solid drugs direct to sunlight during the daytime for 2 days. Degradation of FFD was observed (5.74%) with degradation product at Rf value 0.48 and 18.37% degradation was observed for FP with an additional peak at Rf value 0.85 (Fig. 12).

### Neutral degradation

The FFD and FP showed two additional peaks when treated in water at 50°C for 30 min. Peaks of degraded products were found at Rf value 0.47 (peak 2; FFD 2) and 0.74 (peak 4; FP 5) (Fig. 13).

### DISCUSSION

The proposed stability indicating HPTLC method provides precise, accurate, and reproducible quantitative analysis for simultaneous estimation of FFD and FP in bulk drug and pharmaceutical dosage form. The method was validated as per the ICH guidelines. The linearity was found to be in the range of 1–3.5 µg/spot and 10–60 µg/spot for FFD and FP, respectively. Percentage RSD of intraday and interday precision was found to be <2% making the method more precise. Degradation study revealed that FFD was most prone to degradation under acid (18%, 30 min) stress followed by the stress conditions such as neutral

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**Table 6: Results of LOD and LOQ**

| Drug  | LOD (µg/spot) | LOQ (µg/spot) |
|-------|---------------|---------------|
| FFD   | 0.3           | 1             |
| FP    | 0.2           | 0.6           |

FFD: Formoterol fumarate dihydrate, FP: Fluticasone propionate, LOD: Limit of detection, LOQ: Limit of quantification

**Table 7: Summary of degradation studies for FFD and FP**

| Stressed condition               | FFD      | FP       |
|----------------------------------|----------|----------|
|                                  | Rf (FFD) | % Degradation | DP of FFD at Rf | Rf (FP) | % Degradation | DP of FP at Rf |
| Acid, 0.1 N HCl at 50°C after 30 min | 0.26     | 18.00     | FFD 1–0.15, FFD 2–0.47 | 0.64    | 13.22     | FP 1–0.72     |
| Base, 0.1 N NaOH at 50°C after 15 min | 0.26     | 9.11      | FFD 3–0.42, FFD 4–0.50 | 0.63    | 18.12     | FP 2–0.78     |
| Oxidative, 3% H2O2 at 50°C after 1 h | 0.27     | 13.06     | FFD 4–0.50 | 0.63    | 11.29     | FP 1–0.72, FP 3–0.81 |
| Photolytic, exposure of solid drugs to sunlight during the daytime for 2 days | 0.27     | 5.74      | FFD 4–0.48 | 0.64    | 18.37     | FP 4–0.85     |
| Neutral, distilled water at 50°C after 30 min | 0.27     | 14.34     | FFD 2–0.47 | 0.64    | 13.22     | FP 5–0.74     |

FFD: Formoterol fumarate dihydrate, FP: Fluticasone propionate, DP: Degradation product
(14.3%, 30 min), base (9.11%, 15 min), oxidative (13.06%, 1 h), and photolytic (5.74%, 2 days). FP showed more degradation in basic (18.12, 15 min) and photolytic (18.3%), 24 h) conditions.

CONCLUSION

The developed method was able to separate the drugs from its degradants and impurities. It can be successfully applied as stability indicating method for combination of FFD and FP. Thus, the reported method is of considerable importance and has sound industrial applicability for quality control and stability analysis of FFD and FP from bulk drug and pharmaceutical dosage form.

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AUTHORS’ CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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