A More Rapid, Sensitive, and Specific HPLC-MS/MS Method for Nifedipine Analysis in Human Plasma and Application to a Pharmacokinetic Study

Abstract

A more rapid, sensitive and specific high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) was developed and validated for the quantification of nifedipine in human plasma, and applied to the pharmacokinetic study of nifedipine in Chinese healthy volunteers. Nifedipine and internal standard (IS) acetaminophen in plasma were extracted with ethyl acetate, separated on a C\textsubscript{18} (150 mm × 4.6 mm, 5 μm) reversed-phase column, eluted with acetonitrile mixed with 5 mM ammonium acetate solution (pH = 6.62) (60:40, v/v), ionized by negative ion pneumatically assisted electrospray and detected in the multiple reaction monitoring mode using precursor → product ions of m/z 354.1 → 222.2 for nifedipine and 150.1 → 107.1 for the IS. A single oral dose of 20 mg nifedipine sustained release tablets and blood samples (4 mL) was collected before and 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 8, 12, 24, and 36 h after administration. The main pharmacokinetic parameters of nifedipine, as T\textsubscript{max}, t\textsubscript{1/2z}, C\textsubscript{max}, AUC\textsubscript{0-36}, AUC\textsubscript{0-∞} were 2.80 ± 0.50 h, 6.78 ± 2.52 h, 6.82 ± 2.53 h, 6.69 ± 2.22 h, 76.69 ± 19.51 (ng/mL), 546.49 ± 162.28 (ng·h/mL), and 564.05 ± 176.74 (ng·h/mL), respectively. The calibration curve was linear over the concentration range of 0.17–102 ng/mL (r\textsuperscript{2} > 0.99, n = 5) with a lower limit of quantification (LLOQ) of 0.17 ng/mL. The intra- and inter-day precision was less than 15 % for all quality control samples at concentrations of 0.42, 6.53 and 81.60 ng/mL and the accuracy (relative error, RE) was −3.92 % to 7.31 % at 3 quality control levels. The specificity, matrix effect, recovery, sensitivity, linearity, accuracy, precision and stability were validated, and can fulfill the requirement of pharmacokinetic study of nifedipine sustained release tablets in Chinese volunteers.

Abbreviations

- LLE liquid-liquid extraction
- MRM multiple reaction monitoring
- SPE solid-phase extraction

Introduction

Nifedipine, 1, 4-Dihydro-2, 6-dimethyl-4-(2-nitrophenyl)-3, 5-pyridinedicarboxylic acid dimethyl ester (Fig. 1a), is a dihydropyridine calcium channel blocker and can be used for treatment of hypertension, angina pectoris and other vascular disorders. Through physically plugging the channel and resulting in decreases of intracellular calcium levels, inhibiting the contraction processes of smooth muscle cells, dilate the coronary and systemic arteries, increase oxygen delivery to the myocardial tissue, decrease total peripheral resistance and systemic blood pressure [1].

Numerous analytical methods included gas chromatography (GC), liquid chromatography (LC) and high performance liquid chromatography (HPLC) have been reported for the determination of nifedipine in biological samples. GC methods often utilize electron-capture detection, flame ionization detection, nitrogen-phosphorus detection, or mass spectrometric (MS) detection [2–4] to provide high sensitivity for nifedipine pharmacokinetic studies, but the method has several drawbacks, including its thermal decomposition under GC condition and time-consuming. LC methods without coupling with MS detection [5,6] were also time-consuming in sample disposition. Furthermore, the linearity range from 10–200 ng/mL did not fulfill the requirement for a much low limit of quantitation (LLOQ) of nifedipine pharmacokinetic studies. While the LC/MS
method based on electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) [7–9] provided an LLOQ of 1.0 ng/mL, each run need a relative long time (8 min) and large volume of plasma samples (1.0 mL). UPLC-MS/MS, with short retention time and much lower limit of quantification of 0.01 ng/mL, each run need a relative long time (8 min) and large volume of plasma samples (1.0 mL). UPLC-MS/MS, with short retention time and much lower limit of quantification of 0.01 ng/mL, each run need a relative long time (8 min) and large volume of plasma samples (1.0 mL).

Table 1 Optimized mass spectrometry parameters for nifedipine and IS.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>precursor/fragment productions</th>
<th>Fragment electric voltage (eV)</th>
<th>collision energy (eV)</th>
<th>EMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifedipine</td>
<td>345.1/222.2</td>
<td>100</td>
<td>5</td>
<td>400</td>
</tr>
<tr>
<td>acetaminophen</td>
<td>150.1/107.1</td>
<td>100</td>
<td>20</td>
<td>400</td>
</tr>
</tbody>
</table>

HPLC/MS/MS instrumentation and chromatographic conditions

The HPLC/MS/MS procedure was performed using an Agilent 1200 series HPLC and an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA). All data were analyzed by software Agilent 6410 Quantitative Analysis version analyst data processing software.

Preparation of calibration standards, internal standard, and quality controls

Nifedipine is a photo labile compound, and a nitroso-pyridine derivative is formed in solution on exposure to visible light, while a nitro-pyridine derivative is generated under ultraviolet light [17]. Therefore, the whole process of the experiment must be operated in dark place.

A 10.2 mg aliquot of nifedipine standard and a 10.1 mg aliquot of acetaminophen were weighed in an analytical balance and transferred to 2 A-grade 10 mL volumetric flasks, dissolved with methanol to obtain nifedipine (1.02 mg/mL) and IS (1.01 mg/mL) mother solutions. Primary stock solutions were diluted with the mobile phase for standard working solutions of nifedipine (102, 10.20, 1.02 μg/mL) and IS was dissolved with mobile phase to get a 1010 ng/mL stock solution. All solutions were stored at 4 °C and dark places, and equilibrated to room temperature before use (approximately 15 min). The calibration curve standard and quality control (QC) samples were freshly prepared with blank plasma by spiking with different working solutions. The calibration samples consist of eight nonzero concentrations (0.17–102 ng/mL) and QC samples were 0.17 (LLOQ), 0.42 (LQC) and 6.53 (MQC), 81.60 ng/mL (HQC) for nifedipine.

Plasma pre-treatment

The aim of sample pre-treatment method should remove interferences from the biological sample and also be reproducible with a high recovery and simple procedure involving a mini-
Method Validation

The assays of nifedipine in human plasma were validated in compliance with the US Food and Drug Administration [18] and Chinese State Food and Drug Administration guidelines for the validation of bioanalytical methods including assay selectivity, linearity, recovery, matrix effects, accuracy, precision and stability.

Selectivity

The specificity of the method was evaluated by comparing chromatograms of 6 different lots of blank human plasma to identify the potential interference of endogenous substances at the HPLC peak region (nifedipine and IS).

Calibration curves

Calibration curves were prepared at 8 different nifedipine concentrations. Each calibration standard was injected in 5 replicates. Calibration curves were typically described by equation $y = ax + b$, where $y$ represents the peak-area ratio of nifedipine to IS, and $x$ represents the plasma concentration of nifedipine. The linearity of the calibration curves was assessed by linear regression with a weighting factor of the reciprocal of the concentration ($1/x^2$). The calibrators for analytes were: 0.17, 0.42, 1.04, 2.61, 6.53, 16.32, 40.80 and 102 ng/mL.

The low limit of quantification (LLOQ) was defined as a signal-to-noise ratio greater than 10 and evaluated by analyzing 5 replicates of spiked plasma samples at the concentration of 0.17.

The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value, while that of LLOQ was ±20%.

Recovery and matrix effect

The mean overall recovery of the nifedipine was determined by comparing the peak areas (extracted plasma standards/post extraction plasma samples). Nifedipine were determined by samples at 3 QC levels (0.42 ng/mL as low, 6.53 ng/mL as medium and 81.60 ng/mL as high) with 5 replicates for each QC level. The recovery of the IS (101 ng/mL) was determined in a similar way. Matrix effect was investigated to ensure selectivity, precision and sensitivity that were not compromised by the matrix screened. Blank plasma samples were extracted and spiked with the nifedipine at 3 QC levels and IS in 5 replicates. The corresponding peak areas were compared to those of standard solutions, and the peak area ratio was defined as the matrix effect.

Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing 5 replicates of nifedipine at 3 different QC levels (0.42 ng/mL, 6.53 ng/mL and 81.60 ng/mL) in human plasma. The inter-assay precision was determined by analyzing the 3-level QC samples on 3 consecutive days. The criteria for acceptability of data were accuracy within 85%–115% from the nominal values and a precision of within ±15% relative standard deviation (RSD) or CV%, but that of LQC is not supposed to exceed ±20%.

Stability

The stability of each analyte in plasma was determined by 3 QC levels in 5 replicates. The stabilities of nifedipine in plasma samples at different concentration were examined under different study conditions, including that the post-extracted samples in the HPLC auto-samples at room temperature (25°C) for 0 h (fresh samples) and 7 h, the stability of analytes in human plasma with three QC levels were stored at −20°C for 7 and 60 days and the stability of analytes in human plasma following two freeze-thaw cycles and three freeze-thaw cycles. The samples were processed as described above and the criterion for acceptability of the data is the same with that for the precision and accuracy.

Pharmacokinetic study

20 healthy Chinese male volunteers were involved and all provided written informed consent. Exclusion criteria included the physical examination and laboratory tests when they were unqualified; a history of cardiovascular, hepatic, renal, psychiatric, neurologic, hematologic, or metabolic disease; drug or alcohol abuse within 2 years before the start of the study; allergic constitution; smoking, a history of drug allergy; had severe low blood volume, orthostatic hypotension, arrhythmias, asthma, and a history of glaucoma, sitting heart rate was less than 60 beats/min; consumption of any prescribed or over-the-counter drugs within 2 weeks before the study; or participation in a similar study within the past 6 months. The protocol was approved by the Ethics committee of the College of Medicine, Shandong University, Jinan, China, and the study was conducted in accordance with the declaration of Helsinki and Chinese Good Clinical Practice guidelines.

Demographic characteristics of 20 Chinese male volunteers (mean (SD) for the overall group included age, 25.39(1.32) years; height, 1.74 (0.06) m; BMI, 22.15 (1.71); weight, 67.06 (8.10) kg; blood pressure, 120/80 mm Hg; heart rate, 75 (10) beats/min; blood volume, 7.5 (0.5) L; consumption of any prescribed or over-the-counter drugs within 2 weeks before the study; or participation in a similar study within the past 6 months).

A single oral dose of either test or reference of 20 mg nifedipine sustained release tablets with 200 mL of water were given at 7:00, and blood samples(4 mL)were collected before and 1, 2, 3, 5, 7, 9, 12, 24 h after administration into sodium heparin (20:1) containing tubes, centrifuged at 5000 rpm for 5 min (2266 g) and plasma subsequently quoted into plastic tubes and stored at −20°C for analysis.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Actual concentration (ng · mL$^{-1}$)</th>
<th>Mean</th>
<th>SD</th>
<th>RSD(%)</th>
<th>RE(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.17</td>
<td>0.18</td>
<td>0.16</td>
<td>0.01</td>
<td>8.4</td>
<td>−3.5</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 2 The analyte response at the LLOQ in human plasma (n = 5).
Drug and Statistic software (DAS 2.0, P. R. of China) was used to fit the compartmental model of nifedipine in human and to calculate its main pharmacokinetic parameters.

Results

Method validation

Selectivity
The interference by endogenous plasma constituents with analytes and IS was assessed by inspection of chromatograms that included typical MRM chromatograms of blank plasma, nifedipine and IS standard, blank plasma spiked with nifedipine and IS, and plasma from a volunteer after administration of nifedipine sustained release tablets (Fig. 2), retention time of nifedipine and IS were 3.65 and 2.25 min, respectively, and no significant interferences were found at the retention times of the analytes and IS.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Matrix effect (%)</th>
<th>Extraction recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.42</td>
<td>93.06 ± 6.02</td>
<td>78.05 ± 2.32</td>
</tr>
<tr>
<td>6.53</td>
<td>95.07 ± 6.18</td>
<td>79.61 ± 4.38</td>
</tr>
<tr>
<td>81.60</td>
<td>100.57 ± 5.73</td>
<td>82.88 ± 6.03</td>
</tr>
<tr>
<td>101(IS)</td>
<td>98.30 ± 5.65</td>
<td>78.77 ± 6.67</td>
</tr>
</tbody>
</table>

Fig. 2 chromatograms of a blank plasma, b standard solution of nifedipine and IS, c blank plasma spiked with nifedipine (32.65 ng/mL) and IS, d plasma sample of subject NO. 8 after 3h of oral administration in period 1.

Table 3 Matrix effect and extraction recovery of nifedipine and IS in human plasma (n=5).

Fig. 3 The graph showing the coefficient of correlation of nifedipine samples (n=40) that every calibration curves has eight concentration levels analyzed by HPLC/MS/MS.
Calibration curve and LLOQ
A calibration curve was established ranging from 0.17 to 102 ng/mL nifedipine in plasma. The calibration curves were regressed using a quadratic equation with a weighting factor of 1/x². The coefficient of correlation of all calibration curves were more than 0.99 (n=5) (Fig. 3). The LLOQ was defined as the lowest concentration on the standard calibration curves with acceptable repeatability and recovery. The analyte response at the LLOQ was should be at least 5 times the response of blank baseline. The LLOQ was evaluated by analyzing five replicates of spiked plasma samples at the concentration of 0.17 ng/mL for each analyte. The precision (RSD %) and accuracy (RE %) were found to be 8.4 % and – 3.5 % in Table 2.

Recovery and matrix effect
The extraction recoveries of nifedipine were 78.05±2.32, 79.61±4.38 and 82.88±6.03 at the concentrations of 0.42, 6.53 and 81.60 ng/mL, respectively (n=5), and IS was 78.77±6.67 (101 ng/mL, n=5). The recoveries of nifedipine and IS were similar and the data proved that the liquid-liquid extraction method was sufficient and was not concentration-dependent. The matrix effect is a matter of notorious fact in electrospray ionization mass spectrometry, which will influence the analyte ionization by signal enhancement or suppression. To reduce the matrix effect, the extraction solvent and compositions and ratio of mobile phase were investigated for finding optimal outcome. The matrix effect was 93.06±6.02, 95.07±6.18 and 100.57±5.73 for nifedipine at the concentrations of 0.42, 6.53 and 81.60 ng/mL, respectively (n=5), and 98.30±5.65 for IS (n=5), suggesting that there was no significant matrix effect in this procedure (Table 3). These indicated that the analytical method could be kept free endogenous substances in human plasma.

Accuracy and precision
The intra-day and inter-day precision and accuracy data for nifedipine in plasma is summarized in Table 4, and they were assessed by the determination of QC samples with 5 replicates for each concentration level on the same day or on 3 consecutive days. Precision was expressed by coefficient of variation (RSD) and accuracy by relative error (RE), and accuracy was expressed by mean and standard deviation (SD). The assay values on both the occasions (intra-day and inter-day) were found to be within the accepted variable limits.

Stability studies
Stability for nifedipine after 0h and 7h in autosampleris were shown in Table 5a, 2 freeze-thaw cycles and 3 freeze-thaw cycles were shown in Table 5b, and freeze-dried at −20°C for 7 and 60 days were shown in Table 5c. The results indicated that the analytes were stable at ambient temperature for 0h and 7h after post extracted, at −20°C for 7 and 60 days, and for 2 and 3 cycles of freezing and thawing. The data conform to the acceptance criteria.

Clinical application
The validated method was applied to a pharmacokinetic study for determination of nifedipine concentration in human plasma after a single oral dose of its sustained release tablets. A 1/C weighted coefficients regression analysis and 2-compartmental model were used to fit the disposition of nifedipine in Chinese volunteers. The mean concentration-time curve is shown in Fig. 4. The main pharmacokinetic parameters are shown in Table 6. The results were found to be within the assay variability limits during the entire process.

Table 4 Intraday and interday precision and accuracy for analysis of nifedipine in human plasma.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>intrainaday(n=5)</th>
<th>interday(n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.42</td>
<td>Mean ± SD (ng/mL)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>0.42</td>
<td>0.41 ± 0.03</td>
<td>6.94</td>
</tr>
<tr>
<td>6.53</td>
<td>6.15 ± 0.23</td>
<td>3.74</td>
</tr>
<tr>
<td>81.60</td>
<td>83.23 ± 3.86</td>
<td>4.63</td>
</tr>
</tbody>
</table>

Table 5a The stability of post-extracted samples in the HPLC auto-samples at room temperature (25°C) for 0h and 7h. (n=5).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nominal concentration (ng/mL)</th>
<th>Mean ± SD (%)</th>
<th>RSD (%)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fresh</td>
<td>0.42</td>
<td>0.43 ± 0.03</td>
<td>7.09</td>
<td>3.87</td>
</tr>
<tr>
<td>sample(0h) 6.53</td>
<td>7.13 ± 0.13</td>
<td>1.78</td>
<td>11.33</td>
<td></td>
</tr>
<tr>
<td>post-extracted sample(7h) 81.60</td>
<td>85.94 ± 5.51</td>
<td>6.41</td>
<td>7.42</td>
<td></td>
</tr>
</tbody>
</table>

Table 5b The stability of nifedipine in human plasma following two freeze-thaw cycles and three freeze-thaw cycles. (n=5).
a poor linearity in positive ionization condition and a good response was found in negative ionization mode. The solutions containing nifedipine and IS were injected directly into the mass spectrometer. Under these conditions, the analytes yielded major ions at m/z 345.1 for nifedipine and m/z 150.1 for IS. Each of precursor ions was subjected to collision-induced dissociation to determine the resulting product ions. The full-scan negative product ion mass spectra of precursor ion spectrum of nifedipine and IS and the product ion mass spectra of nifedipine and IS are shown in Fig. 5. The results showed that the most sensitive and abundant mass transitions were m/z 345.1 → 222.2 for nifedipine and 150.1 → 107.1 for IS. The MRM state file parameters were the optimized values for the sensitivity and specificity required for nifedipine.

Selection of IS
It was difficult to find a compound that could ideally mirror the analyte to serve as a suitable IS that should mimic the analyte during extraction and have a stationary response, especially, for HPLC-MS/MS, matrix effect used to induce poor analytical. Several compounds were investigated, such as nitrendipine, diazepam, hydrochlorothiazide, glycyrrhetinic acid and acetaminophen. Nitrendipine had long retention time, diazepam was no peak under the negative ion detection mode, hydrochlorothiazide had serious smear under the conditions used and glycyrrhetinic acid did not proper product ion under product scan mode. None of these, but finally acetaminophen (Fig. 1b) was found to be the most appropriate for the present purpose. The retention time of acetaminophen was short to that of nifedipine. Chromatograms were obtained and no significant direct interference in the MRM channels at the relevant retention time was observed and it could save time for a large samples analysis.

Sample pre-treatment
Protein precipitation, Liquid-liquid extraction (LLE) and solid phase extraction (SPE) were tested and compared, with acetonitrile, toluene, and ether-n-hexane (3:1, v/v) as protein precipitation solvents, but protein precipitation was easy to dilute the sample and failed to sufficiently remove endogenous interference. SPE had too many disturbances to good reproducibility and recovery [16, 19]. What is more, the column for SPE is expensive and not suitable for high-throughput analysis when a large number of samples were processed. LLE was used for producing chromatography clean samples in the study, which contributed to minimizing ion suppression and matrix effects in HPLC-MS/MS. LLE with various extraction solvents, including diethyl ether, chloroform, dichloromethane and ethyl acetate, were investigated and evaluated for acceptable extraction recoveries and matrix effect. Diethyl ether, extraction recoveries were about 42.12 % and unacceptable, chloroform was easy to emulsify when the sample was made vortex and mixed and its recover was about 54.20 %, dichloromethane, matrix effects were about 75.23 % and disturb the results. Ethyl acetate was with several obviously advantages, firstly, the upper organic phase was transferred easily, secondly, ethyl acetate possesses less toxicity, good stability and repeatability, finally, extraction recoveries were approximately 81.63 % (Table 7). Furthermore, it was also tested whether sodium hydroxide (0.05 mol/L) was added to extraction solvents, and there were not obvious difference for peak area and retention time.
Solvents included methanol, acetonitrile, 5mmol/L ammonium acetate and mobile phase for reconstituting residues were also investigated to optimize the chromatographic behaviors for optimizing peak shape and minimum peak response. Mobile phase with good peak shape and minimum response was adopted.

Liquid chromatography
A simple chromatographic separation was developed for acquisition of good separation with a short run time. The feasibility of various mixtures of solvents such as acetonitrile and methanol using different water phase, including 5 mM ammonium acetate (pH=6.62), 2 mM ammonium acetate (pH=7.19), water and 1% methanoic acid were tested, along with altered flow-rates (in the range of 0.5–1 mL/min), was tested to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. It was found that acetonitrile mixed with 5mM ammonium acetate solution (pH=6.62) (60:40, v/v) could achieve this purpose in negative ionization mode and finally used as the mobile phase. A flow rate of 0.8 mL/min permitted a run time of 5 min.

Conclusions
In summary, a highly sensitive, specific, reproducible and high-throughput HPLC-MS/MS method that we developed and validated based on the procedure of LLE for determination of nifedipine with IS. The procedure was fully validated to meet the requirements for sensitivity, accuracy and precision from State Food and Drug Administration and GLP Guidelines for industry. According to the validation parameters, the developed method could be useful for nifedipine pharmacokinetic studies and routine therapeutic drug monitoring with desired precision and accuracy.

Table 7 The development summary of liquid-liquid extraction procedure (20 ng/mL, n = 5).

<table>
<thead>
<tr>
<th>extraction solvent</th>
<th>Matrix effect Mean ± SD (%)</th>
<th>RSD (%)</th>
<th>Extraction recovery Mean ± SD (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>diethyl ether</td>
<td>71.53 ± 9.63</td>
<td>11.32</td>
<td>42.12 ± 5.76</td>
<td>19.77</td>
</tr>
<tr>
<td>chloroform</td>
<td>68.11 ± 2.11</td>
<td>10.97</td>
<td>54.20 ± 6.89</td>
<td>8.15</td>
</tr>
<tr>
<td>dichloromethane</td>
<td>75.23 ± 5.22</td>
<td>14.33</td>
<td>68.42 ± 8.71</td>
<td>17.88</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>97.56 ± 5.12</td>
<td>5.40</td>
<td>81.63 ± 5.10</td>
<td>4.32</td>
</tr>
</tbody>
</table>

Fig. 5 full-scan negative product ion mass spectra of a precursor ion spectrum of nifedipine; b product ion spectrum of nifedipine; c precursor ion spectrum of IS; d product ion spectrum of IS.

Acknowledgements
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Conflict of Interest
The authors declare no conflict of interest.

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