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Original Article

DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING ISOCRATIC REVERSE PHASE-LIQUID CHROMATOGRAPHY ASSAY FOR DETERMINATION OF PHENYTOIN IN BULK AND PHARMACEUTICAL FORMULATIONS

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ABSTRACT

Objective: To develop and validate a stability-indicating reversed phase high performance liquid chromatography (RP-HPLC) assay for the determination of phenytoin in bulk and pharmaceutical dosage forms.

Methods: A HPLC instrument incorporating aZorbaxC-18 analytical column (250x4.6 mm, 5µm particles) with a mobile phase comprising acetonitrile: water in the ratio 50:50 (%v/v) was employed for the determination of phenytoin. The flow rate was set with an isocratic program, the temperature of the column was maintained at 25 °C and a detection wavelength of 200 nm was employed using an ultraviolet detector. The method was validated as per The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines.

Results: Total chromatographic analysis time per sample was 6 min with phenytoin eluting with the reaction time of 4.6±0.2 min. Phenytoin was exposed to acidic, basic, oxidative, photolytic and thermal stress conditions and the specificity of the assay was confirmed. The calibration plot was linear (R^2≥0.999) over the phenytoin concentration range 5.0-100.0µg/ml. The percentage means recoveries were found to be in the range of 98-102%. The relative standard deviation of precision and robustness were within prescribed limits (<2%). The limit of detection was 0.047 µg/ml while the limit of quantitation was established as 0.143 µg/ml.

Conclusion: A simple, accurate, precise and stability-indicating RP-HPLC assay was successfully developed for the determination of phenytoin in bulk and dosage forms. Hence, this assay is useful for the analysis of phenytoin in formulations in medicines development and pharmaceutical manufacturing setting.

Keywords: Phenytoin, RP-HPLC, Stability-indicating assay, Forced degradation, Validation.

INTRODUCTION

Phenytoin (5, 5-diphenylhydantoin or Dilantin) was first introduced as an anticonvulsant medication in 1937[1]. It helps to dampen the un wanted, runaway brain activity associated with seizures by stabilizing the inactive state of voltage-gated sodium channels, leading to a reduction in electrical conductance among brain cells. Phenytoin is a member of the family of phenyl hydantoins (fig. 1), exhibiting a pK_a value of 8.3 and is practically insoluble in water [2]. Phenytoin is normally administered as a solid oral dosage form (capsules) in the treatment of epilepsy [3] and has been employed both topically and systemically in the treatment of lichen planus, epidermolysis bullosa and inflammatory conditions [4]. A number of studies have also shown that phenytoin has a positive effect on wound healing in a variety of cases including leg ulcers, leprosy wounds, burns and diabetic foot ulcers [4-7].

Despite the proven clinical utility of phenytoin, there are relatively few analytical methods reported for the determination of phenytoin in bulk, in formulations and in blood plasma. These are generally based on thin layer chromatography, spectrophotometry, enzyme immunoassay, radioimmunoassay and liquid chromatography [8-13]. Athin layer chromatographic method was used to determine phenytoin in pharmaceutical formulations and identify its hydroxylated urinary metabolites [8]. A novel study reported the development of a liquid chromatography method coupled with electro spray ionization tandem mass spectrometry to detect and quantify phenytoin in human plasma [9]. Walash *et al.* (2011) developed a spectrophotometric method for detection and determination of trace impurities in phenytoin bulk powder and pharmaceutical formulations [10]. The quantitative assay of phenytoin in sera by various methods, such as spectrophotometry, gas chromatography, liquid chromatography, enzyme immunoassay and radioimmunoassay had been described [11]. Khe dr *et al.* (2008) reported a HPLC method for determination of phenytoin in rabbits receiving sildenafil [12]. Lin *et al.* (2010) measured free and total levels of phenytoin in human plasma collected from patients with epilepsy using micellar electro kinetic chromatography method [13]. However, the methods for quantitative determination of phenytoin in pharmaceutical formulations are very scarce in the scientific literature.

Hence, the objective of this study was to develop a simple, specific, accurate, precise, sensitive and stability-indicating isocratic RP-HPLC assay for the determination of phenytoin in bulk and pharmaceutical dosage forms. This method was validated in accordance with ICH guidelines [14-16].

MATERIALS AND METHODS

Chemicals and reagents

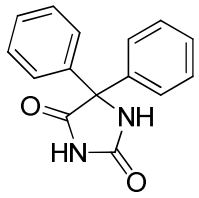

Fig. 1: Chemical structure of phenytoin

Phenytoin was purchased from Chemolab Sdn. Bhd. (Selangor, Malaysia). Phenytoin capsules were obtained from Pfizer Inc. (New York, United States). Acetonitrile of HPLC grade was purchased from Fisher Scientific Sdn. Bhd. (Selangor, Malaysia). Other chemicals of HPLC grade purity were purchased from commercial sources. Water was purified using Sartorius Ultrapure Water System (Goettingen, Germany).

HPLC method and chromatographic conditions

Chromatographic system and conditions

Analysis of phenytoin in bulk (non-formulated powder) and in formulations was performed using a HPLC system (1200 series, Agilent Technologies) equipped with a pump, injector valve with 20 µl sample loop, ZORBAX Eclipse Plus C-18 analytical column (250×4.6 mm, 5 µm particles) and ultraviolet (UV) detector with data processor (Chem Station Software). UV detection for phenytoin was set at 200 nm. The mobile phase acetonitrile: water (ACN: H_2O) of 50:50 (%v/v)was delivered at ambient temperature (25±2 °C) at a flow rate of1.0 ml/min. Before analysis, the mobile phase was filtered through a 0.45 µm, 47 mm membrane filter (Membrane Solution, United States) and degassed in an ultrasonic bath (Fisher Scientific, Malaysia). The mobile phase was delivered by an isocratic method under RP-HPLC conditions and the system was equilibrated before each injection.

Preparation of standard stock solution

A standard stock solution was prepared by dissolving phenytoin powder (5 mg, accurately weighed) in 5 ml acetonitrile (final concentration 1 mg/ml). The prepared solution was retained in an ultrasonic bath for 30 min and filtered through a 0.22 µm, 13 mm syringe filter (Membrane Solution, United States). Standard calibration (or working solutions) was prepared by diluting aliquots of the stock solution to obtain concentrations ranging from 5-100 µg/ml.

Sample preparation from phenytoin labeled capsules

The contents of ten capsules (30 mg phenytoin-labeled capsules) and five capsules (100 mg phenytoin-labeled capsules) were individually weighed. Acetonitrile (10 ml) was added to aportion of the powder, equivalent to 10 mg phenytoin, and the solution was retained in an ultrasonic bath for 30 min. An aliquot of this solution was diluted with acetonitrile to obtain a phenytoin concentration of 50 µg/ml. The solutions were filtered through a 0.22 µm, 13 mm syringe filter (Membrane Solution, United States) before injection into the HPLC instrument.

HPLC method development andoptimization

Five different wavelengths of ultraviolet light (225, 220, 215, 201 and 200 nm) were investigated in the development of the HPLC method suitable for analysis of phenytoin in bulk and in formulations. The effect of each wavelength on the peak area was examined. A range of acetonitrile: water mobile phase compositions were investigated, namely 60:40 55:45, 50:50, 45:55 and 40:60 (%v/v). Final selection of the mobile phase composition was decided on the basis of time required for analysis, ease of preparation, the sensitivity and specificity of the assay.

System suitability

Testing was performed to ensure that the HPLC assay was suitable for the intended analysis. Ten samples of the standard solution containing phenytoin at 45 µg/ml were analyzed to determine the chromatographic parameters of peak area, retention time, capacity factor, tailing factor and number of theoretical plates.

Method validation

Specificity

Specificity refers to the ability to measure the molecule of interest in the presence of other components that may exist in the sample. Interference with measurement of the molecule of interest may be occasioned by other active entities, excipients, impurities, and degradation products and must be eliminated to ensure that a peak response is only due to a single component. Specificity was assessed by analyzing non-formulated (bulk) phenytoin alone and phenytoin removed from the capsules. The specificity of the method and the stability-indicating potential were confirmed by analyzing samples of bulk and formulated phenytoin that had been subjected to forced degradation by exposure to sunlight, ultraviolet radiation, and heat, and hydrogen peroxide, acidic and basic conditions. The various stress conditions are presented in the table 1. All stress treatment was carried out at 25 °C apart from heat stress testing at 60 °C. Following treatment, phenytoin solutions were prepared of concentration 50 µg/ml and subsequently analyzed by HPLC. The analyte peak was evaluated both for peak purity and the potential peak interference.

Table 1: Stress conditions applied prior to assay of phenytoin

Stress type	Condition	Time (h)
Photolytic degradation	Sunlight	24
	Ultraviolet light	1
Thermal	60 °C	24
Oxidative	0.3% H_2O_2	24
Acidic hydrolysis	0.1NHCl, pH 1	24
Basic hydrolysis	0.1NNaOH, pH 13	24

Linearity

Calibration standards for phenytoin were prepared from stock solution (1 mg/ml) at concentrations of 5, 10, 15, 20, 40, 50, 60, 80 and 100 µg/ml. A calibration plot was constructed of peak area against concentration.

Limit of detection and limit of quantification

The limit of detection (LOD) is the lowest detectable amount of analyte in a sample whereas the limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be quantified. The LOD and LOQ were calculating dusing the following equations as per ICH guidelines.

$$LOD = 3.3 \text{ X } \frac{\sigma}{S} \rightarrow \text{Eq. 1}$$

$$LOQ = 10 \text{ X } \frac{\sigma}{S} \rightarrow \text{Eq. 2}$$

Where S is the slope of the calibration plot and σ is the standard deviation of the response for the blank samples.

Accuracy

Accuracy of measurement was established using a recovery (spiking) study, in which known amounts of phenytoin corresponding to five concentration levels (50%, 80%, 100%, 120% and 150%)of a reference concentration (50µg/ml) were injected into the HPLC instrument. The experiment was performed in triplicate. The measured concentration of phenytoin in the injected samples by HPLC analysis was determined by measuring the peak area and fitting these values to the linear equation of the peak area versus concentration calibration curve.

The recovery, bias and RSD values were determined in order to ensure the accuracy of the assay. The recovery percentage and bias percentage were calculated for each concentration level using following equations:

$$\text{Recovery (\%)} = \frac{C_a}{C_t} \times 100 \rightarrow \text{Eq. 3}$$

$$\text{Bias (\%)} = \frac{C_a - C_t}{C_t} \times 100 \rightarrow \text{Eq. 4}$$

Where C_t and C_a are theoretical concentration and the actual concentration of injected samples, respectively.

Precision

Precision was determined as both repeatability and intermediate precision, in accordance with ICH recommendations. Repeatability of measurements was determined as intra-day variation by analyzing samples within a day. Intermediate precision was determined by measurement of inter-day variation following a daily analysis of samples for three days. For both intra-day and inter-day precision, solutions of phenytoin (six samples) of concentration 50 µg/ml were analyzed.

Robustness

The robustness of the developed method was investigated to assess the effect of small but deliberate variations of chromatographic conditions on the assay of phenytoin. Robustness was assessed by changing the flow rate (0.9 and 1.1 ml/min), the mobile phase composition (ACN: H_2O=48:52% v/v and 52:48%v/v) and the column temperature (23 °C and 27 °C).

Stability of the analytical solution

The stability of phenytoin in solution during HPLC analysis was determined by repeated analysis of samples during the course of experimentation on the same day and also after storage of the drug solution for one week under ambient conditions (25±2 °C) and under refrigeration (4±2 °C) respectively.

Application of the developed HPLC method to marketed formulations of phenytoin

The HPLC method developed in this study was applied to estimate the phenytoin content of commercially available 30 mg phenytoin-labeled capsules and 100 mg phenytoin-labeled capsules.

RESULTS AND DISCUSSION

HPLC method development and optimization

In the development of analytical assays, optimal conditions are established based on speed, reliability and reproducibility. In this work, no internal standard was used because no extraction or separation step was involved. Prior knowledge of the UV absorption spectra of phenytoin assisted the initial development of the assay. The maximum absorption (peak area) of phenytoin in acetonitrile was previously reported as 200 nm [13]. The maximum absorption of a 20 µg/ml phenytoin solution in acetone in the present study also occurred at a wavelength (λ_{max}) of 200 nm (table 2). In addition and as expected, the peak area was found to be affected by the wavelength used.

Table 2: Peak area versus UV wavelength for phenytoin solutions using HPLC

Sample	Peak area				
	225 nm	220 nm	215 nm	201 nm	200 nm
1	649.60	1059.60	1639.50	3671.90	3894.50
2	666.90	1054.80	1607.80	3603.10	3869.40
3	652.90	1073.60	1636.90	3624.90	3964.20
Mean	656.47	1062.67	1628.07	3633.30	3909.37
SD	9.18	9.77	17.60	35.16	49.12
RSD	1.40	0.92	1.08	0.97	1.26

The results of the relationship between the composition of the mobile phase and the resulting chromatographic profiles for phenytoin assay are presented in table 3. Most compositions did not furnish sharp, well-defined peaks and either resulted in much lower sensitivity or did not give well-defined peaks in a short time scale. ACN: H_2O ratio of 50:50 (%v/v) was found to furnish sharp, well-defined peaks with the best symmetry (0.94) combined with lower tention time (4.6 min). Hence, the wavelength of 200 nm and the mobile phases ACN: H_2O ratio of 50:50 (%v/v) were used in the present study.

Table 3: The effect of mobile phase composition on HPLC assay of phenytoin

Mobile phase composition (ACN: H_2O)	Retention time (min)	Peak area	Symmetry
60:40	3.49	9425.47	0.91
55:45	3.92	9300.83	0.88
50:50	4.57	10014.50	0.94
45:55	5.64	9610.13	0.79
40:60	7.47	9590.23	0.73

System suitability

System suitability was evaluated by replicate injections (n=10) of a standard solution containing phenytoin at 45µg/ml. The results of retention time, peak area, capacity factor, tailing factor, and number of theoretical plates for the analyte are presented in Table 4. The RSD values of retention time and peak area were within 1%, indicating the suitability of the system. The capacity factor, USP tailing factor and the number of theoretical plates were within the acceptance criteria of>1, ≤1.5 and>2000, respectively [17]. These results thus indicated high efficiency and suitability of the developed HPLC system for the intended application.

Table 4: Analysis of HPLC system suitability for the assay of phenytoin

Properties	Mean (n=10)	RSD (%)
Retention time	4.57	0.08
Peak area	8947.34	0.98
Capacity factor	3.59	0.13
Tailing factor	0.79	1.39
Number of theoretical plates	6152.70	1.43

Method validation

Specificity and forced degradation study

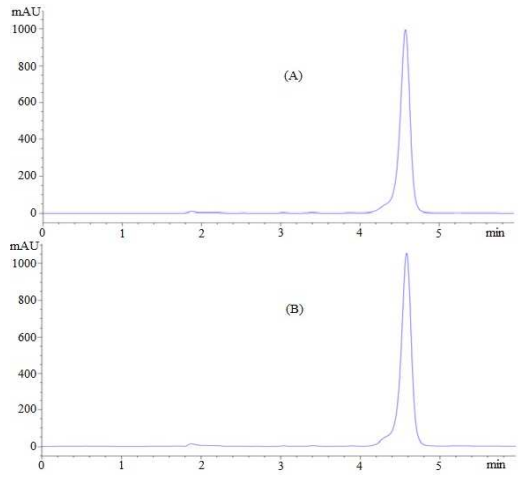

Fig. 2: Typical HPLC chromatograms of standard (bulk) phenytoin (A) and phenytoin extracted from capsule formulations (B)

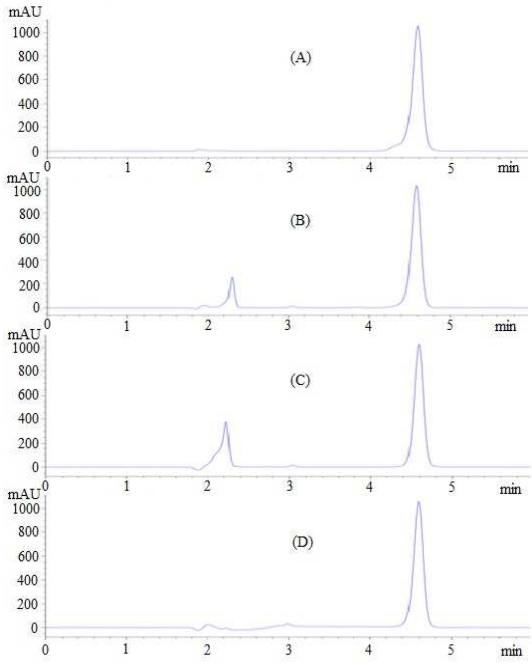

Fig. 3: Typical chromatograms of phenytoin solution subjected to forced degradation (A) sunlight, UV lightor elevated temperature of 60 °C (B) oxidative condition(C) basic condition (D) acidic condition

The specificity of the developed HPLC method was assessed by analyzing phenytoin in its non-formulated or bulk powder form (fig. 2A) and phenytoin extracted from capsule formulations (fig. 2B). The peaks obtained were well resolved from the solvent front and no interference was observed, indicating that the method was specific for phenytoin in bulk and in formulations.

The specificity and stability-indicating potential of the developed HPLC method was confirmed by exposing a solution of phenytoin to different stress conditions. The drug demonstrated relatively good stability under heat and photolytic (sunlight and UV light) conditions.

The retention time and peak area of these chromatograms was similar to untreated samples (fig. 3A). Phenytoin was found to degrade under oxidative condition (fig. 3B) and alkaline condition (fig. 3C) giving rise to small peaks at retention times of 2.3 min and 2.2 min, respectively. The chromatogram of phenytoin following exposure to acidic condition (fig. 3D) revealed minor peaks occurring at a retention time of 1.8-2.3 min,

In all forced degradation studies carried out in this study, there was no significant change in peak area and retention time of the active drug. The peaks of the degradation products were completely resolved from the peaks for phenytoin and did not interfere with drug analysis. Thus, the developed method offers potential for the selective and specific determination of phenytoin in pharmaceutical formulations.

Linearity & range

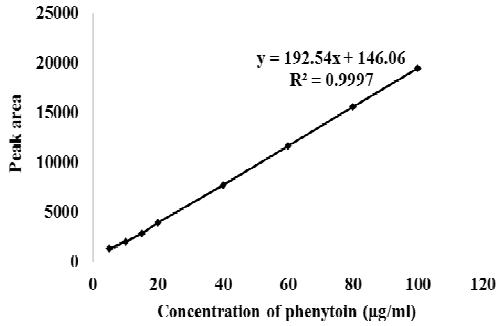

Fig. 4: Linearity and range of the HPLC method developed for assay of phenytoin

A standard curve for phenytoin (fig. 4) was constructed by plotting the peak area versus drug concentration of phenytoin over the concentration range of 5-100 µg/ml. The goodness-of-fit (R^2) was found to be 0.9997, which indicated a good linear relationship between the concentration of the analyte and the peak area.

The calibration data, with their relative standard deviations (RSD) are listed in table 5. The low values of RSD (<2%) confirm that the method is precise and the calibration plot does not deviate from linearity.

Table 5: Relationship between peak area and concentration of phenytoin solution using HPLC analysis

Concentration (µg/ml)	Peak area±SD, (n =3)	RSD (%)
5	1354.40±13.03	0.96
10	2047.57±38.71	1.89
15	2933.20±25.69	0.88
20	3925.17±34.49	0.88
40	7766.97±45.16	0.58
60	11628.33±29.24	0.25
80	15588.73±180.06	1.16
100	19462.63±107.56	0.55

Limit of detection and limit of quantification

The LOD and LOQ of phenytoin in the mobile phase were determined to be 0.047 and 0.143 µg/ml, respectively. Both findings indicate that the developed method is sensitive and can be used for detection and quantification of phenytoin over a wide range of concentrations.

Accuracy

The percentage recovery of phenytoin at each concentration level (50%, 80%, 100%, 120% and 150%) is presented in table 6. The percentage recovery (measured amount relative to the actual injected amount) was within acceptable limits (100±2%). Furthermore, the bias and RSD were within±2%. All the results confirmed that the developed HPLC method was accurate for the determination of the phenytoin.

Precision

Repeatability of measurement was determined as intra-day variation while inter-mediate precision was determined by measuring inter-day variations for six samples of phenytoin at 50 µg/ml. Repeatability and intermediate precision (table 7) were found to be within the limits prescribed in ICH guidelines (RSD<2%).

Table 6: Accuracy of developed HPLC method for phenytoin

Spike level (%)	Concentration of drug added (µg/ml)	Measured concentration of drug (µg/ml)	Mean recovery (%)	SD	RSD (%)	Bias (%)
50	25	24.96	99.85	0.34	1.37	0.15
80	40	39.93	99.83	0.31	0.79	0.17
100	50	49.94	99.89	0.43	0.86	0.11
120	60	60.62	101.03	0.41	0.68	1.03
150	75	75.02	100.03	0.92	1.22	0.03

Table 7: Intra-day and inter-day precision of developed HPLC assay of phenytoin

Sample	Intra-day precision			Inter-day precision
	Day 1	Day 2	Day 3	
Sample-1	10006.60	9934.10	9918.00	
Sample-2	9770.80	9967.30	10046.90	
Sample-3	9850.80	9927.70	9970.80	
Sample-4	10009.40	9951.60	10168.80	
Sample-5	9917.40	9880.60	10167.80	
Sample-6	9828.90	9918.40	10207.60	
Mean	9897.32	9929.95	10079.98	9969.08
SD	97.73	29.85	119.28	97.42
RSD (%)	0.99	0.30	1.18	0.98

Robustness

The robustness of the developed method was established by monitoring the influence of small changes in chromatographic conditions on peak area and retention time. No significant changes (table 8) in these parameters were detected (RSD<2%) when changes were made to the organic content of the mobile phase (±2%), temperature (±2 °C) and flow rate (±10%).

Thus minor modifications to the experimental parameters do not affect the developed assay and its ability to accurately and precisely detect or quantify phenytoin.

Solution stability

The results presented in table 9 demonstrate that the analytical phenytoin solution (45 µg/ml) remains stable for one week at room temperature (25±2 °C) and during refrigeration (4±2 °C) respectively with no significant degradation. All RSD values were less than 2%.

Assay of phenytoin content in capsule formulations

The developed HPLC method was applied to measure the content of phenytoin in commercially available capsule formulations. The mean percentage recovery of phenytoin in 30 mg and 100 mg phenytoin-labeled capsules was found to be 99.23±1.75%and 99.13±1.63% respectively, in close agreement with the labeled amounts. Corresponding RSD values are within the limits prescribed in ICH guidelines (RSD<2%).

These findings suggest that the developed HPLC method is highly suitable for the accurate determination of phenytoin in pharmaceutical formulations.

Table 8: The influence of changes in chromatographic parameters on HPLC analysis of phenytoin

Parameters	Modification	Retention time (min)	RSD (%)	Peak area	RSD (%)
Normal condition	-	4.58	0.07	9805.80	0.51
Mobile phase composition, ACN: H_2O(%v/v)	48:52	4.93	0.03	9816.20	0.46
	52:48	4.29	0.08	10000.27	0.53
Temperature(°C)	23	4.53	0.12	9846.53	1.05
	27	4.50	0.01	9686.00	0.62
Flow rate (ml/min)	0.9	5.09	0.03	11132.93	0.89
	1.1	4.14	0.47	9995.67	1.15

Normal conditions: ACN: H_2O=50:50 (%v/v); temperature 25 °C, Flow rate 1.0 ml/min.

Table 9: Stability of phenytoin solutions revealed by the developed HPLC assay

Storage conditions	Freshly prepared phenytoin solution			Phenytoin solution stored for one week		
	Retention time (min)	Peak area	RSD (%)	Retention time (min)	Peak area	RSD (%)
25±2 °C	4.57	8939.17	0.66	4.56	8831.43	0.41
4±2 °C				4.57	8908.97	1.22

CONCLUSION

An isocratic RP-HPLC assay was developed for the determination of phenytoin and validated according to ICH guidelines. The method has been proven to be accurate, precise, reproducible, specific and stability-indicating. Furthermore, the assay utilizes an economical and readily available mobile phase and has been found to exhibit linearity between peak area and drug concentration over a wide concentration range. All these factors recommend the HPLC method for quantification of phenytoin in the bulk,non-formulated state and in pharmaceutical dosage forms.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest

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