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Original Article

DEVELOPMENT AND VALIDATION OF HPLC-UV METHOD FOR SIMULTANEOUS ANALYSIS OF ACRYLAMIDE AND GLYCIDAMIDE IN VOLUMETRIC ABSORPTIVE MICROSAMPLING

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ABSTRACT

Objective: Acrylamide is a carcinogenic compound that can be found in commonly consumed foods and cigarette smoke. This compound is metabolized by cytochrome P450 in the human body to a more reactive metabolite, glycidamide. This study aimed to optimize and validate a sensitive HPLC-UV method for determining acrylamide and glycidamide simultaneously in the volumetric absorptive microsampling (VAMS) sample.

Methods: Isoniazid as an internal standard was added to the VAMS sample containing acrylamide and glycidamide prior to protein precipitation. The analytes and internal standard were separated using reversed-phase chromatography with the C18 Sunfire™Waters® column (5 µm; 250 mm x 4.6 mm) and an ultraviolet detector.

Results: The optimum chromatographic condition was eluted at a column temperature of 30 °C with a mobile phase of 6 mmol potassium dihydrogen phosphate pH 3.5–methanol (96:4 v/v) using a flow rate of 0.50 ml/min and was detected at 210 nm. The LLOQ was obtained at 1.0 µg/ml for both acrylamide and glycidamide. The calibration curve was linear over the concentration range of 1.0-100.0 µg/ml.

Conclusion: The developed bioanalytical method was valid based on US FDA Guideline for Bioanalytical Method Validation 2018.

Keywords: Acrylamide, Glycidamide, HPLC-UV, Volumetric absorptive microsampling, Validation

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INTRODUCTION

Cancer is one of the major causes of death around the world. The incidence and mortality of cancer are fast-growing. According to the Global Cancer Observatory's (Globocan) 2018 database, there were more than 18 million new cancer cases and 9.5 million cancer-related deaths. In the year 2020, the new cancer case increased to 19.29 million cases and 9.95 million people died of cancer [1]. Approximately 29% of cancer deaths are caused by cigarette smoking [2]. One of the carcinogenic compounds in cigarette smoke is acrylamide (ACR). Besides, acrylamide is also found in coffee and commonly consumed carbohydrate-rich foods such as potato crisps, French fries, cereal products, crackers, and biscuits. Acrylamide is also reported as neurotoxic, genotoxic, reproductive toxic, hepatotoxic, immunotoxic [3], and cardiotoxic [4]. Acrylamide is formed by the Maillard reaction which is the nonenzymatic reaction between the amino acid asparagine and reducing sugars at high temperatures. After consumption, acrylamide is rapidly absorbed by the gastrointestinal and converted by cytochrome P450 (CYP450) to its active metabolite, glycidamide (GLY). Glycidamide has been known to be more reactive to DNA and protein to form DNA and protein adducts than acrylamide. Acrylamide intake that causes cancer was estimated to be 2.6 mg/kg body weight/day [3-6].

To assess the risk of ACR and GLY exposure in humans, it is important to measure the ACR and GLY levels in the blood. Determination of ACR and GLY has been done in several previous studies, using different bio-sampling techniques which are venipuncture [7] and dried blood spot (DBS) [8]. Collecting blood by venipuncture is more invasive, requires the phlebotomist, and the plasma sample obtained needs a refrigerator to keep the sample stable. Collecting blood by DBS has several advantages such as using the capillary blood sample from a finger which is less invasive than venipuncture and easier to take the sample without phlebotomist requirement. In addition, the dried sample is more stable thus it can be stored at room temperature without the refrigerator. However, DBS has some disadvantages such as hematocrit bias and homogeneity [9].

Currently, several bioanalytical methods using volumetric absorptive microsampling (VAMS) have been developed to overcome DBS's problems. VAMS is the newer bio-sampling technique that allows for collecting a fixed volume (10, 20, and 30 µl) of a blood sample onto a porous hydrophilic tip. VAMS has the same advantages as DBS due to using the capillary blood sample followed by drying at room temperature. Moreover, the tip of the VAMS sampler is designed to absorb blood accurately and precisely thus minimizing hematocrit bias and homogeneity [10–12].

High-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector is used in this study due to this instrument is relatively less expensive than LC-MS/MS which is used in previous studies. Therefore, the purpose of this study is to develop and validate the bioanalytical method of ACR and GLY using VAMS as the more precise bio-sampling technique and HPLC-UV as the more economical instrument.

MATERIALS AND METHODS

Materials

ACR, GLY, and isoniazid (ISO) were purchased from Sigma-Aldrich (Singapore), and volumetric absorptive microsampling was purchased from Neoteryx® (Torrance, CA, USA). Methanol, acetonitrile (HPLC grade), phosphoric acid 85% (analytical grade), and potassium dihydrogen phosphate were purchased from Merck (German). Aquabidest was purchased from Ikapharmindo (Indonesia) and human whole blood was obtained from Indonesian Red Cross/Palang Merah Indonesia (Indonesia).

Instrument

The HPLC system was conducted using the LC-20A Shimadzu series with an autosampler and UV detector (Shimadzu, Japan). Analysis was performed on Waters® Sunfire™C18 column (5 µm; 250 mm x 4.6 mm) (Waters, USA), and detected at 210 nm.

Preparation of stock and working solutions

The stock solutions of ACR, GLY, and ISO were prepared by dissolving 10 mg each of ACR, GLY, and ISO separately in 10 ml of distilled water. The working standard solutions were prepared by diluting the stock solutions in distilled water.

Preparation of calibration standards and quality control (QC) samples

The calibration standards of ACR and GLY were prepared by spiking an appropriate amount of the working solutions into whole blood followed by dipping the tip of VAMS samplers into the spiked blood and VAMS absorbed 30 µl of spiked blood. The concentration range of ACR and GLY in the calibration curve was 1,0-100,0 µg/ml and quality control (QC) samples were prepared separately at three concentrations that were quality control low (QCL) of 3,0 µg/ml), quality control medium (QCM) of 50,0 µg/ml), and quality control high (QCH) of 100,0 µg/ml).

Chromatographic condition optimization

The optimized parameters were molarity and pH of buffer solution as a part of the mobile phase, the composition of the mobile phase, flow rate, and column temperature. The tested variation of buffer solution was 6 mmol, 8 mmol, and 10 mmol for molarity, and 3.5, 4.0, and 4.5 for the pH. The tested variation of mobile phase composition was 92:8, 94:6, and 96:4 (v/v). The flow rate tested was 0.5, 0.6, and 0.7 ml/min. The variation of column temperature that was tested was 30, 35, and 40 °C [13].

System suitability test

After the optimum chromatographic condition was obtained, system suitability was tested to ensure that the HPLC system is fit to use and working precisely.

Sample preparation optimization

VAMS sampler was dipped into the spiked blood containing ACR and GLY with appropriate concentrations, then dried for 2 h at room temperature. Dried VAMS samples were extracted by removing the tips from plastic handles and putting them into microtubes followed by adding 100 µl of ISO 10,0 µg/ml as the internal standard and then extracting solvent. The extracting solvents used for optimization were methanol 100%, methanol-acetonitrile (50:50 v/v), and acetonitrile 100%. The variation of extracting solvent volume used for optimization was 500, 750, and 1000 µl. Then, the mixture was vortexed, sonicated, and centrifugated. The time variations were 30, 60, and 90 seconds for vortex, 5, 10, and 15 min for sonication, and 1, 3, and 5 min for centrifugation. The supernatant was evaporated under nitrogen at 40 °C for 20 min. The dried sample was reconstituted in 100 µl of distilled water and then sonicated for 15 min, vortexed for 30 seconds, and centrifugated for 5 min at 3000 rpm. Finally, 20 µl of aliquot was injected into the HPLC system [8].

Sensitivity

The sensitivity value is defined as the lower limit of quantification (LLOQ) which is the lowest concentration that meets precision and accuracy requirements with the coefficient of variation (CV) value of ≤ 20% and the relative difference (% diff) of the measured and actual value within±20%. Analysis of half of the previous concentration can be carried out if the previous results meet the requirement [14].

Selectivity

The selectivity test was evaluated by determining 2 replicates of LLOQ and blank samples from 6 different sources. The acceptance criteria for the selectivity test is that the interference response at the retention time of the analyte is≤ 20% of the LLOQ response and≤ 5% of the internal standard response [14].

Carry-over

Carry-over was assessed by analyzing the blank after analysis of the upper limit of quantification (ULOQ) concentration. Analysis was carried out for 5 replicates. Interference from blanks at the retention time of the analyte was observed. The response of the interference must be≤ 20% of the LLOQ response and ≤ 5% of the internal standard response [14].

Calibration curve and linearity

The calibration curve was evaluated by determining a minimum of 6 concentration levels, blank and zero samples. Analysis was carried out 3 times. The linear equation was created by plotting the PAR (peak area ratio) of the analyte to the internal standard (y) versus concentrations (x). The linear equation was used to recalculate the calibration standard concentration. The recalculated concentration should be within±15% of the actual concentration, except for LLOQ should be within±20% of the actual concentration [14].

Precision and accuracy

Precision and accuracy were evaluated by determining LLOQ, QCL, QCM, and QCH with 5 replicates on the same day (within-run) and the different day (between-run). The requirement for within-and between-run precision was less than 15%, and the inaccuracy (bias) was within±15%, except for LLOQ was within 20% [14].

Recovery

Recovery is a comparison between the response of extracted samples and blank extract spiked after extraction. The recovery test was carried out for 3 replicates of QC samples. The acceptance criteria are reproducible with a CV value not exceeding 15% [14].

Dilution integrity

Dilution integrity was tested for 5 replicates of twice concentrations of QCH or above ULOQ that were serially diluted to within the concentration range of the calibration curve. The dilution integrity was acceptable if inaccuracy values (bias) is within±15% and precision (CV) is not exceeding 15% [14].

Stability

The stock solution and VAMS sample stability were evaluated by comparing the measurement results of the samples stored at temperatures of 25 °C and 4 °C for a certain time to the measurement results of the freshly prepared samples. The stability of VAMS samples was carried out at two concentrations (QCH and QCL) in three replicates. The value obtained must be less than 2% for the stock solutions and less than 15% for VAMS samples [14].

RESULTS

Chromatographic condition optimization

The optimum molarity and pH of buffer solution were 6 mmol and 3.5, respectively, with the mobile phase composition of 96:4 (v/v) using the flow rate of 0.5 ml/min. The optimum column temperature was obtained at 30 °C.

System suitability test

According to the optimum condition, the average retention time of GLY, ACR, and ISO was 5.52 min, 9.69 min, and 17.22 min, respectively. The total run time was 20 min. The chromatogram was shown in fig. 1. The CV value of peak area was 0.25%, 0.34%, and 0.21% for GLY, ACR, and ISO, respectively. The CV value (%) of retention time was 0.87%, 0.70%, and 0.74% for GLY, ACR, and ISO, respectively.

Sample preparation optimization

The optimum extracting solvent was acetonitrile 100% with a volume of 500 µl. The optimum time for vortex, sonication, and centrifugation were 30 sec, 5 min, and 1 min, respectively.

Sensitivity, calibration curve, and linearity

Analysis at a concentration of 0.5 µg/ml resulted in accuracy value (% diff) for GLY between-227.25% to-162.56% with a CV value of-28.24% and % diff for ACR between 65.47% to 79.96% with a CV value of 3.29%. Analysis at 1.0 µg/ml gave a % diff value ranged from-6.22% to 14.89% for GLY and from 7.29% to 16.19% for ACR. The CV value was 8.89% and 3.17% for GLY and ACR, respectively. Therefore, the result of sensitivity was that 1,0 µg/ml was chosen as the LLOQ. The calibration curves showed good linearity in the concentration range of 1,0-100,0 µg/ml with a correlation coefficient (r) for GLY between 0.9990 to 0.9994 and ACR between 0.9992 to 0.9993.

Precision and accuracy

The results of within-and between-run accuracy calculated from a three-day validation period showed that the % diff value for GLY was in the range of-11.56% to 13.31% with a precision (CV) value

less than 6.93%. The % diff value for ACR was in the range of-12.79% to 13.05% with a precision (CV) value being less than 9.72%. The results were shown in table 1. The chromatograms of QC samples (low, medium, and high) were shown in fig. 2.

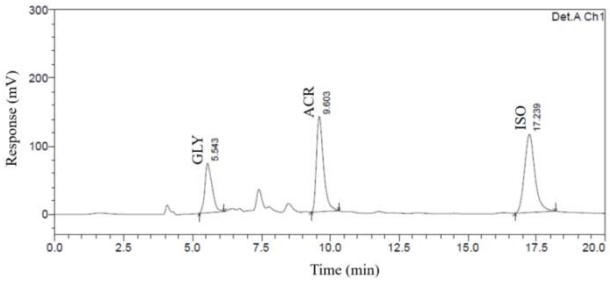
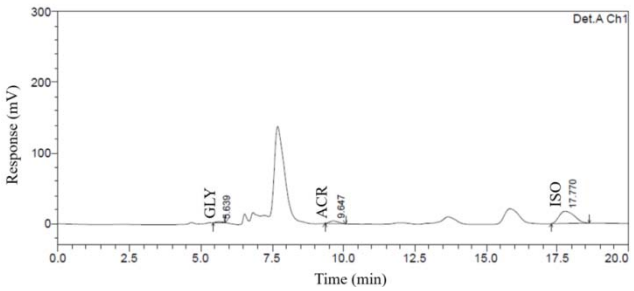


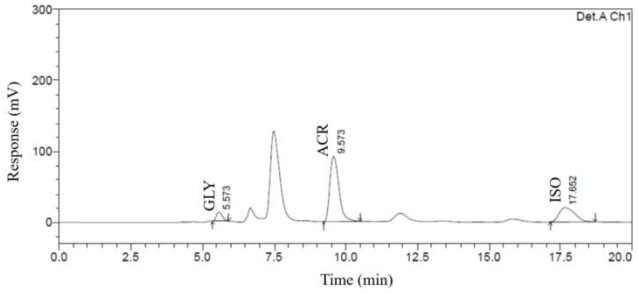
Fig. 1: The chromatogram of the system suitability test

Table 1: The result of precision and accuracy within-and between-run

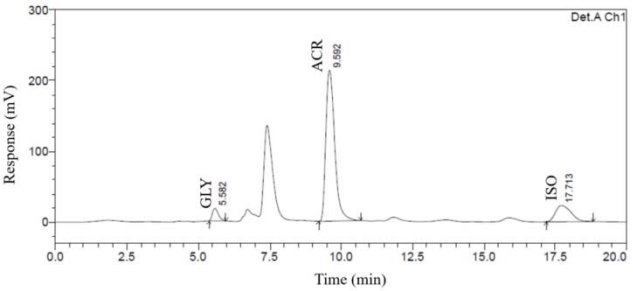
	Conc. (µg/ml)	Within-run		Between-run	
		Precision (% CV)	Accuracy (% diff)	Precision (% CV)	Accuracy (% diff)
GLY	1.0	6.09	-11.56 to 3.20	4.96	-11.56 to 11.40
	3.0	6.93	-7.57 to 10.21	1.78	-9.53 to 10.21
	50.0	4.16	1.39 to 12.19	2.55	-8.32 to 12.19
	75.0	3.67	1.05 to 9.77	3.45	-11.13 to 13.31
ACR	1.0	0.89	10.54 to 13.05	5.96	-3.05 to 13.05
	3.0	2.26	4.58 to 10.52	4.32	-5.31 to 10.52
	50.0	3.19	-4.80 to 3.54	2.96	-4.80 to 10.70
	75.0	9.72	1.05 to 9.77	5.09	-12.79 to 11.40



(a)



(b)

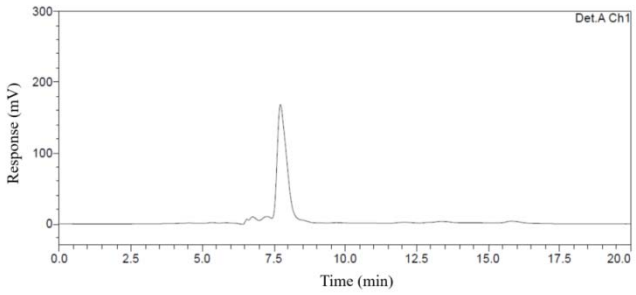


(c)

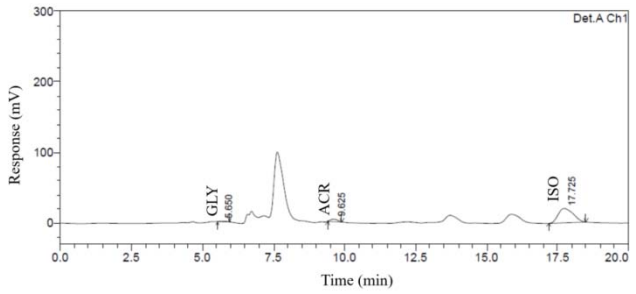
Fig. 2(a-c): The chromatogram of (a) QCL, (b) QCM, and (c) QCH

Selectivity and carry-over

The value of the selectivity test showed that the interference response at the retention time of GLY was less than 0.77%, ACR was less than 0.26%, and there was no interference at the retention time of ISO. The chromatograms of blank and LLOQ were shown in fig. 3. The results of the carry-over test showed that the interference response at the retention time of GLY was less than 0.97%, ACR was less than 3.08%, and there was no interference at the retention time of ISO.



(a)



(b)

Fig. 3(a-b): The chromatogram of (a) blank and (b) LLOQ

Recovery and dilution integrity

The mean extraction recovery of GLY at the concentrations of QCL, QCM, and QCH were 93.49%, 90.99%, and 92.35%, with a CV value of 1.53%, 2.46%, and 1.63%, respectively. Whereas, for ACR were 94.09%, 96.37%, and 92.61%, with the CV value of 1.98%, 3.85%, and 4.51%. The dilution integrity test resulted in the % diff value ranging from-7.70% to 9.47% for GLY and from-11.37% to-2.37% for ACR. The CV value was less than 5.69% and 3.97%, for GLY and ACR, respectively.

Stability

According to the results of the stability test, the % diff value for samples stored in the refrigerator (4 °C) was in the range of-9.69 to 9.83 for GLY and-11.68% to 10.00% for ACR. The results of the long-term stability test in the room (25 °C) and the post-preparation stability test in the autosampler (25 °C) also showed that the % diff value was less than 15%. The stability test results of GLY and ACR were shown in table 2.

Table 2: The result of the stability test

	Stability	Period	QCL (3,0 µg/ml)		QCH (75,0 µg/ml)	
			Mean measured conc. (µg/ml)±SD (n=3)	% diff	Mean measured conc. (µg/ml)±SD (n=3)	% diff
GLY	Short term (25 °C)	24 h	2.98±0.07	-2.57 to 1.86	79.38±2.61	3.42 to 9.83
	Long term (25 °C)	21 d	3.02±0.12	-3.74 to 4.44	79.50±0.88	4.65 to 6.70
	Long term (4 °C)	21 d	2.72±0.01	-9.69 to-9.22	73.17±0.64	-3.37 to-1.70
	Autosampler (25 °C)	24 h	2.98±0.15	-7.55 to 2.05	78.50±1.02	3.11 to 5.67
ACR	Short term (25 °C)	24 h	2.71±0.05	-11.08 to-7.91	68.95±2.24	-11.51 to 6.09
	Long term (25 °C)	21 d	3.06±0.20	-2.16 to 10.00	67.43±1.88	-11.68 to-7.20
	Long term (4 °C)	21 d	2.84±0.19	-10.90 to 1.48	69.82±3.93	-10.75 to-0.94
	Autosampler (25 °C)	24 h	3.04±0.12	-3.16 to 4.75	70.29±5.87	-11.78 to 2.68

DISCUSSION

Chromatographic condition optimization was performed to obtain maximum peak area, good retention time, and good peak shape. The tailing factor increase as the molarity and pH of the buffer solution increase. Increasing molarity also increases the column pressure that affects the column effectiveness during analysis. Therefore, the chosen molarity and pH of the buffer solution were 6 mmol and 3.5, respectively. Increasing the composition of methanol in the mobile phase, flow rate, and column temperature significantly decreases the retention time and resolution. However, there were some peaks between glycidamide and acrylamide peaks which were unknown impurities in the blood. The carboxyl group or ester bond and a double bond from endogenous compounds are known that absorb UV light at 210 nm [15] which was the wavelength used for detection in this method. Methanol as the mobile phase also absorbs UV light in the range of 190 to 210 nm [15] so the chromatogram of the system suitability test (fig. 1) showed some noise between glycidamide and acrylamide peak. The too fast retention time was not good due to the impurity peaks can disturb the analyte peaks.

Therefore, the mobile phase composition used was buffer solution-methanol 96:4 (v/v), the flow rate was 0.5 ml/min, and the column temperature was 30 °C. The analysis conditions were tested by the system suitability test with a CV value of less than 2%. It can be concluded that the analysis conditions used were eligible.

Sample preparation optimization was started with the selection of extracting solvent. Methanol 100% extract provided many impurities at the retention time of analytes and internal standard. Interferences at the retention time of analytes were also shown in extraction using a mixture of methanol-acetonitrile (50:50 v/v). However, acetonitrile 100% as extracting solvent provided good selectivity, cleaner chromatogram, and less interference appeared at the retention time of analytes and internal standard. In addition, acetonitrile 100% had a benefit as compared to methanol-water (1:1) used in the reference sample preparation (8). Acetonitrile is easier to be evaporated rather than the mixture of methanol-water (1:1) so it can be evaporated at a lower temperature for a shorter time. Therefore, acetonitrile 100% was selected to extract VAMS samples. The volume of extraction has also been optimized, and the volume of 500 µl was able to extract analytes optimally. Then, the mixture was vortexed, sonicated, and centrifugated. Vortexing and sonicating are the important process that helps to draw analytes from the tip of the VAMS sample. Centrifugation is required to separate impurities through the centrifugal force applied by rotating the sample tube very quickly. Vortexing for 30 seconds, sonicating for 5 min, and centrifuging for 1 min were the most effective and efficient times. A longer time to vortex, sonicate, and centrifuge did not result in a larger peak area of analytes.

Method validation was carried out to ensure that the developed bioanalytical method was selective, sensitive, accurate, reproducible, and suitable for the analysis of the study samples [14]. The results of the selectivity test showed that the method can distinguish between analytes and internal standard with impurities. The developed method met the selectivity requirement that the interference peak area at the retention time of the analytes is less than 20% of the LLOQ peak area and the interference peak area at the retention time of the internal standard is less than 5%.

The carry-over test was carried out to detect the appearance of analytes and internal standard in a sample from a previous injected sample. The results of the carry-over test showed that the interference peak area at the retention time of the analyte is less than 20% of the LLOQ peak area and the interference peak area at the retention time of the internal standard is less than 5% so that it was acceptable according to FDA guidelines.

The sensitivity test resulted in the LLOQ or lowest analytes concentration that can be accuracy (% diff≤ 20%) and precision (CV ≤ 20%) was 1.0 µg/ml for both analytes. While the concentration of 0.5 µg/ml was not acceptable based on FDA guidelines.

The calibration curve used seven points of concentration, which are 1, 2, 5, 10, 25, 50, 100 µg/ml, blank and zero sample. The correlation coefficient was in the range of 0.9990-0.9994, which means that the curve was linear and the % diff of the measured concentration met the requirements.

Within-and between-run accuracy and precision data were within±20% for LLOQ and±15% for QC samples as well as CV not exceeding±20% for LLOQ and 15% for QC samples. The results showed the analytical method was accurate and precise thus fulfilling the acceptance criteria of FDA guidelines.

A high recovery value (≥ 90%) with CV ≤ 15% showed that the sample extraction process was able to extract optimally and reproducible. A dilution integrity test was conducted to ensure that the dilution of the sample is accurate, precise, and reliable. The dilution process is required to analyze the *in vivo* samples containing analytes that exceed the ULOQ concentration. The results showed that the analytical method fulfilled the requirements of the dilution integrity test.

The results of the stability test showed that the stock solutions and VAMS samples were stable in the refrigerator (4 °C) for 21 d. In-room (25 °C), the stock solutions were stable for 24 h while VAMS samples were stable for 21 d. Post-preparation samples were stable in autosampler (25 °C) for 24 h.

CONCLUSION

The developed bioanalytical method has fulfilled all the full validation parameters based on FDA 2018 guidelines, which are sensitivity, calibration curves and linearity, accuracy, precision, selectivity, carry-over, recovery, dilution integrity, and stability. The method was linear over the concentration range of 1,0-100,0 µg/ml with a correlation coefficient of 0.9990-0.9994.

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Nil

AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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