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Original Article

DEVELOPMENT AND VALIDATION OF A GC-HS-FID METHOD FOR DIMETHYL SULFATE IN CLOBAZAM API: INTEGRATION OF (Q)SAR-BASED GENOTOXICITY ASSESSMENT

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ABSTRACT

Objective: To develop and validate a sensitive gas chromatography–headspace method with flame ionisation detection (GC–HS–FID) for trace-level quantification of dimethyl sulfate (DMS) in clobazam active pharmaceutical ingredient (API), supported by an in silico quantitative structure-activity relationship ((Q)SAR) based genotoxicity assessment in accordance with International Council for Harmonisation (ICH) M7(R1).

Methods: DMS was derivatised to anisole using phenol under alkaline conditions in a dimethylformamide–water diluent and analysed by GC–HS–FID. The method was validated in accordance with ICH Q2(R1) guidelines. Mutagenicity was evaluated using VEGA (Q)SAR models within their applicability domains.

Results: The method demonstrated high specificity with no interference at the anisole retention time. Linearity was observed over the studied concentration range (R^2 = 0.9993). The limit of detection and limit of quantification were 0.04 ppm and 0.12 ppm, respectively. Precision and accuracy met ICH acceptance criteria, and the method remained robust under minor variations in chromatographic conditions. In silico (Q)SAR analysis consistently predicted dimethyl sulfate as mutagenic within the applicability domain.

Conclusion: The validated GC–HS–FID method is sensitive and reliable for routine monitoring of dimethyl sulfate in clobazam API. The in silico (Q)SAR assessment corroborates its classification as a class 1 genotoxic impurity (GTI), supporting the need for stringent control.

Keywords: Dimethyl sulfate, Genotoxic impurity, ICH M7, Quantitative structure–activity relationship, Clobazam, Gas chromatography-headspace, Trace analysis

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INTRODUCTION

Dimethyl sulfate (DMS) is a highly reactive electrophilic methylating agent widely used in organic and pharmaceutical synthesis to introduce methyl groups onto nucleophilic sites such as hydroxyl, amino, and thiol functionalities [1, 2]. Owing to its strong alkylating reactivity, DMS is a well-established genotoxic and carcinogenic compound, as demonstrated in regulatory and toxicological evaluations [3-5].

Unlike volatile residual solvents regulated under ICH Q3C(R8) [6], DMS is classified under International Council for Harmonisation (ICH) M7(R1) as a Class 1 genotoxic impurity (GTI), reflecting clear evidence of direct DNA reactivity and mutagenicity in both *in vitro* and *in vivo* models [3, 5, 6]. Clobazam is a 1,5-benzodiazepine used as an anticonvulsant in the management of epilepsy and related seizure disorders. Synthetic routes to clobazam utilise DMS as a methylating agent for key intermediates [7, 8], potentially allowing trace levels of residual DMS to persist as a process-related impurity in the final active pharmaceutical ingredient (API). The chemical structures of DMS and clobazam (fig. 1) highlight the presence of a potent GTI in a chronically administered central nervous system drug, underscoring the need for sensitive analytical control.

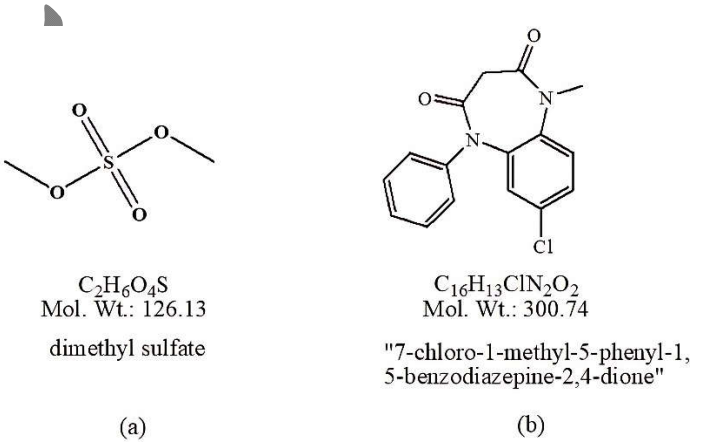


Fig. 1: Structure of dimethyl sulfate (a) and clobazam (b)

Analytical determination of DMS is challenging due to its high volatility, lack of a chromophore, poor direct response in gas chromatography with flame ionisation detection, and susceptibility to hydrolysis in aqueous or mildly basic media [9]. To address these limitations, derivatisation approaches converting DMS into more stable and detectable derivatives, such as phenolic methylation to anisole, have been reported [10, 11], and gas chromatography–headspace (GC–HS) techniques have been shown to improve sensitivity for volatile impurities [9]. Several analytical approaches, including derivatisation-based gas chromatography, gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) with derivatisation, and headspace techniques, have been reported for DMS in pharmaceutical matrices [9, 12–19]; however, these methods are not specifically optimised for trace-level determination of DMS in clobazam API for routine quality-control use.

In line with ICH M7(R1) expectations for impurity risk assessment [20], in silico (Q)SAR tools were applied to support the genotoxic classification of DMS using validated Ames mutagenicity models implemented within the VEGA platform [21].

The objective of this study was to develop and validate a gas chromatography–headspace flame ionisation detection method for trace-level quantification of dimethyl sulfate in clobazam API, supported by an in silico (Q)SAR-based genotoxicity assessment in accordance with ICH Q2(R1) and ICH M7 guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Phenol (≥99.9%, analytical reagent grade (AR), Thermo Fisher, USA), potassium hydroxide (≥99%, AR, SDFCL, India), and N,N-dimethylformamide (GC–headspace grade, ≥99.9%, Thermo Fisher, USA) were used for preparation of the derivatisation diluent. Dimethyl sulfate (≥99.5%, AR, AVRS Synthesis, India) was used for preparation of standard solutions. High-performance liquid chromatography (HPLC) grade water was obtained from an ELGA purification system. Clobazam active pharmaceutical ingredient (API) samples were provided by Flowchem Pharma Pvt. Ltd., India.

Regulatory justification and calculation of the acceptable DMS limit

The target analytical limit for dimethyl sulfate (DMS) was established in accordance with International Council for Harmonisation (ICH) M7(R1). The acceptable intake (AI) of DMS specified as 1.5 µg/day was normalised to the maximum daily dose (MDD) of clobazam (60 mg/day). The corresponding permissible concentration in the active pharmaceutical ingredient (API) was calculated as follows: acceptable concentration (ppm) = (1.5 µg/60,000 µg) × 10^6 = 25 ppm. This calculated value (25 ppm) was used as the target concentration for method development and to define the calibration range (8–150% of the working level).

Instrumentation

Gas chromatographic analysis was performed using a gas chromatography system (GC-2010 Pro, Shimadzu, Japan) equipped with a flame ionisation detector (FID) and a headspace autosampler (HS-20, Shimadzu, Japan). Analyses were conducted using 20 ml headspace vials. Chromatographic separation was achieved using a DB-5 capillary column (30 m × 0.25 mm internal diameter, 1.0 µm film thickness; Agilent Technologies). All weighing operations were carried out using an analytical balance (AP225WD, Shimadzu, Japan) with a readability of 0.1 mg. Volumetric preparations were performed using calibrated pipettes and volumetric flasks.

Derivatisation strategy and GC–HS–FID method principle

A gas chromatography–headspace method with flame ionisation detection (GC–HS–FID) was employed for the quantification of dimethyl sulfate (DMS), considering its high volatility, lack of a UV chromophore, and limited direct detectability by conventional chromatographic techniques. DMS undergoes electrophilic methyl transfer via an SN2 mechanism, as commonly observed in phenolic methylation reactions, resulting in the formation of volatile methyl ether derivatives suitable for headspace gas chromatographic analysis [2, 19]. An indirect derivatisation approach was therefore applied to enable sensitive and reproducible GC analysis.

In this approach, phenol was deprotonated using potassium hydroxide (KOH) to generate potassium phenoxide, which reacts quantitatively with DMS to form anisole. Potassium hydroxide was selected in preference to sodium hydroxide (NaOH) because of its higher solubility and more complete dissociation in the *N,N*-dimethylformamide–water system, resulting in improved derivatisation efficiency and chromatographic performance. The anisole formed is a stable and volatile derivative suitable for headspace GC–FID analysis, and its quantification serves as an indirect measure of DMS content in the sample matrix. The derivatisation reaction scheme is shown in fig. 2.

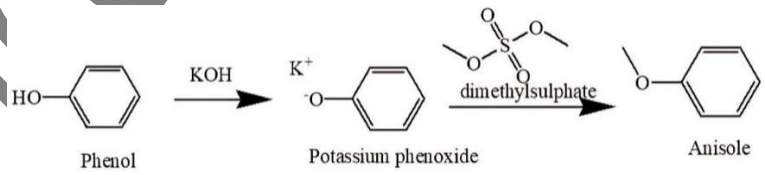


Fig. 2: Reaction scheme of dimethyl sulfate derivatisation to anisole

GC–HS–FID analytical conditions

Chromatographic separation was carried out on a DB-5 capillary column. The oven temperature programme was set with an initial hold at 80 °C for 4 min, followed by a ramp of 5 °C/min to 100 °C, and a second ramp of 20 °C/min to 230 °C, which was maintained for 5 min. The detector temperature was maintained at 240 °C. The column flow rate was set at 3.24 ml/min, with a total flow of 19.4 ml/min and a split ratio of 1:5.

Headspace conditions were optimised to ensure efficient volatilisation and transfer of the derivatised analyte. Samples were equilibrated at 90 °C for 20 min. The sample loop temperature was maintained at 110 °C and the transfer line temperature at 120 °C. The injection time was 1.00 min, with loop equilibration and fill times of 0.10 and 0.20 min, respectively. The vial pressurisation time was set to 1.0 min, with a pressure equilibration time of 0.4 min. The total GC cycle time was 28 min.

Diluent, standard, and sample solutions preparation

Preparation of diluent

The derivatisation diluent was prepared by accurately weighing 5.0 g of phenol (0.212 M) and 2.5 g of potassium hydroxide (0.178 M) into a 250 ml volumetric flask. The contents were diluted to volume with a premixed solution of N,N-dimethylformamide and water in a 1:1 (v/v) ratio. The solution was sonicated for 1 min and shaken for an additional 1 min to ensure homogeneity. The final pH of the diluent was approximately 12.3.

At this pH, greater than 99% of phenol is present in its deprotonated form (pKa ≈ 10), ensuring sufficient availability of phenoxide ions for quantitative derivatisation of trace-level DMS while avoiding excessive free hydroxide.

Preparation of standard stock and calibration solutions

A primary DMS stock solution was prepared by dissolving 15.0 mg of DMS in the diluent and making up to 100 ml (150 µg/ml). An intermediate stock was obtained by diluting 2.0 ml of the primary stock to 100 ml with diluent (3.0 µg/ml; equivalent to 3.0 ppm in the headspace vial).

Appropriate dilutions of the intermediate stock were prepared to obtain calibration levels at 0.12, 0.38, 0.75, 1.20, 1.50, 1.80, and 2.25 ppm (8–150% of the target concentration of 1.5 ppm). For headspace analysis, 2.0 ml of each calibration solution was transferred into a 20 ml headspace vial and sealed with a PTFE-lined septum and aluminium crimp cap.

Preparation of working standard solution

A working standard was prepared by diluting 50 ml of the 3 ppm intermediate stock to 100 ml with diluent. An aliquot of 2.0 ml of this solution was placed into a 20 ml headspace vial and sealed with a Polytetrafluoroethylene (PTFE)-lined septum and aluminium crimp cap.

Sample and blank preparation

For sample preparation, approximately 200 mg of clobazam active pharmaceutical ingredient (API) was weighed directly into a 20 ml headspace vial, followed by the addition of 2.0 ml of the derivatisation diluent. The vial was immediately sealed with a polytetrafluoroethylene (PTFE)-lined septum and aluminium crimp cap to minimise analyte loss due to volatilisation.

A blank solution was prepared by transferring 2.0 ml of the derivatisation diluent into a 20 ml headspace vial and sealing it in the same manner.

In silico (Q)SAR models for genotoxicity assessment

To support the regulatory classification and risk assessment of DMS in accordance with ICH M7(R1) guidelines, an in silico (Q)SAR-based evaluation was conducted using the VEGA QSAR Platform (version 1.3.18) [19] (https://www. vegahub. eu/portfolio-types/*in silico*-tools/). This software integrates multiple QSAR models to predictmutagenicity**.** The chemical structure of DMS, represented by the Simplified Molecular Input Line Entry System (SMILES) notation O=S(=O)(OC)OC, was assessed for mutagenic potential using a battery of predictive models. For mutagenicity (Ames test)**,** the following models were employed: CONSENSUS model (version 1.0.4)**,** CAESAR (2.1.14)**,** ISS (1.0.3)**,** SARpy-IRFMN (1.0.8)**,** and KNN-Read-Across (1.0.1)**.** Model predictions were evaluated with respect to applicability domain status, reliability, and the presence of structural alerts. In line with ICH M7 Section 2.3.2, complementary expert rule-based and statistical models were employed, and all predictions were reviewed using expert judgement. Concordant positive predictions from at least two validated models within their applicability domains were considered sufficient to classify DMS as mutagenic.

RESULTS

A comparison of reported analytical methods for the determination of dimethyl sulfate is presented in table 1.

Table 1: Reported analytical methods for the determination of dimethyl sulfate in pharmaceuticals and related matrices

S. No.	Compound name	Analytical method	Detection range	Reference
1	API Salt (API name not disclosed)	liquid–liquid extraction followed by gas chromatography–mass spectrometry (GC-MS)	LOD: S/N ratio>5 lOQ: 0.1 ppm	[9]
2	Cephalosporin	headspace-solid-phase microextraction (HS-SPME)/GC-MS	0.25 to 4 µg/ml	[12]
3	Pantoprazole sodium sesquihydrate	derivatisation followed bythe UPLC/MS method	LOD: 1.94×10^{-7} mg/ml, LOQ: 6.46×10^{-7} mg/ml.	[13]
4	4-(2-methoxyethyl)phenol	derivatisation followed by LC-MS-based detection.	LOQ: 0.05 µg/ml, linear upto 10 µg/ml	[14]
5	Lipophilic bulk drug	GC (wide-bore capillary)	Not specified	[15]
6	Hexaconazole	headspace GC-MS	0.035 mg/kg to 9.234 mg/kg	[16]
7	API Intermediate (methyl sulfate salt).	liquid–liquid extraction and GC-MS	LOD: 0.3 ppm, LOQ: 1 ppm	[17]
8	API (not specified)	capillary zone electrophoretic, indirect UV detection method	LOD: 0.12 µg/ml LOQ: 0.36 µg/ml	[18]
9	Methoxsalen API	pre-column derivatisation with static GC-HS	LOD: 2 µg/g LOQ: 5 µg/g	[19]
10	Clobazam API	derivatisation followed by GC-HS-FID	LOD: 0.04 ppm LOQ: 0.12 ppm	This study

Abbreviations: GC–MS: gas chromatography–mass spectrometry; HS–SPME: headspace solid-phase microextraction; UPLC–MS: ultra-performance liquid chromatography–mass spectrometry; GC–HS–FID: gas chromatography–headspace flame ionisation detection; LOD: limit of detection; LOQ: limit of quantification; API: active pharmaceutical ingredient.

Optimisation of derivatisation time

A time-dependent derivatisation study was performed at 90 °C, and the anisole peak response was recorded at equilibration times of 5, 10, 15, 20, and 25 min. The peak area increased from 2,718 at 5 min to 14,487 at 20 min and showed minimal change at 25 min (14,478). The percentage relative standard deviation (% RSD) across all time points was ≤ 0.12%. The corresponding data are summarised in table 2.

The formation of anisole was verified by LC-MS. The derivatised sample analysed in negative electrospray ionisation mode showed a characteristic ion at m/z 129.10 in (A) part of (fig. 3). When the anisole reference standard was analysed in positive electrospray ionisation mode, it produced a sodium-adduct ion at m/z 130.45 ([M+Na]+) in (B) part of (fig. 3). Although the ion observed in the negative mode does not represent a sodium adduct, the agreement in chromatographic behaviour and the complementary mass spectral signatures between the derivatised sample and the reference standard confirm successful conversion of DMS to anisole.

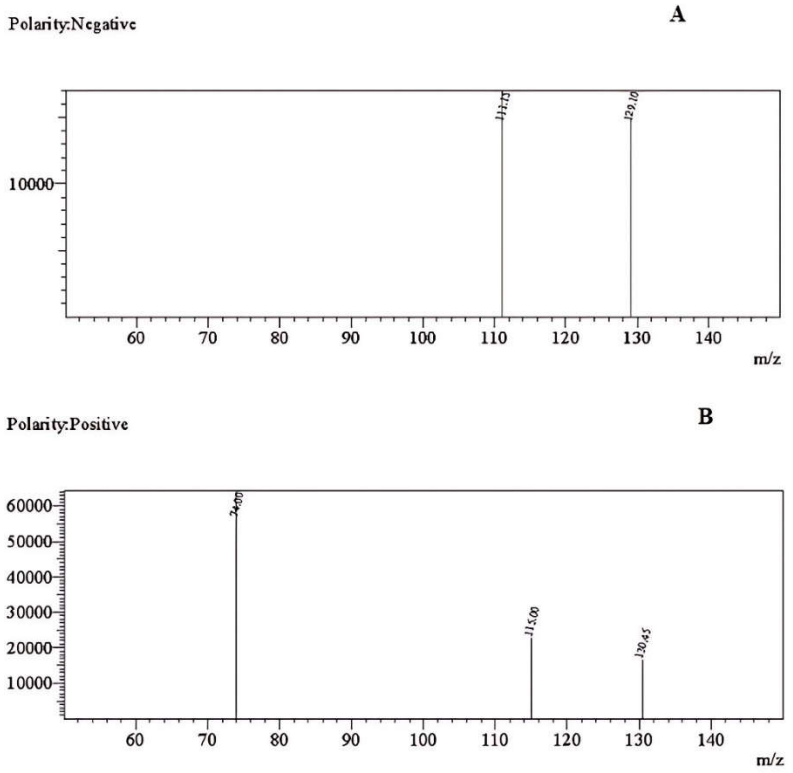


Fig. 3: LC–MS spectra confirming the formation of anisole after derivatisation of DMS. (A) Negative-ion-mode spectrum of the derivatised sample showing a characteristic ion at m/z 129.10, (B) Positive-ion-mode spectrum of the anisole standard showing a sodium-adduct ion at m/z 130.45 ([M+Na]+)

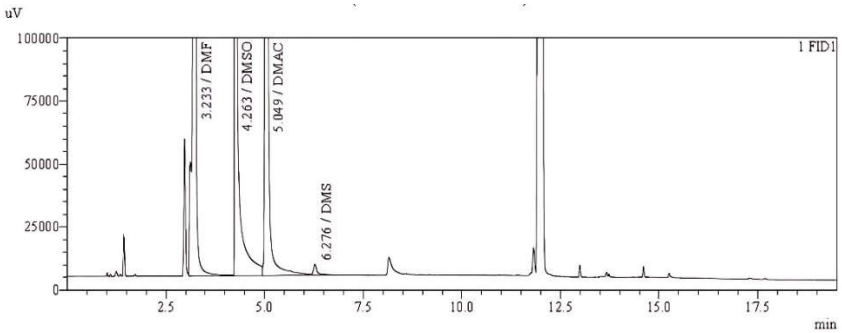
The effect of equilibration time on peak response was tabulated in table 2.

Table 2: Effect of equilibration time at 90 °C on anisole peak area

Time (min)	Mean peak response	SD	% RSD
5	2718	3.22	0.12
10	6747	4.51	0.07
15	9111	2.52	0.03
20	14487	4.04	0.03
25	14478	3.61	0.02

value represent n = 3. SD: standard deviation; RSD: relative standard deviation, several GC columns were evaluated for chromatographic performance. The DB-624, Rtx-225, and DB-1 columns produced broad or tailing peaks, whereas the DB-5 column produced sharp and symmetrical peaks with improved resolution.

Different diluents were examined for their chromatographic behaviour under the optimised GC–HS–FID conditions. The retention times of N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and N,N-dimethylacetamide (DMAc) were observed at approximately 3.2, 4.2, and 5.0 min, respectively, while the anisole peak eluted at approximately 6.2 min (fig. 4). Among the evaluated diluents, DMF showed a resolution of 10.5 between the DMF and anisole peaks.



Peak Table

FID1

Peak#	Name	Ret. Time	Area	Area%	Resolution(USP)
1	DMF	3.233	4448703	45.203	--
2	DMSO	4.263	1984309	20.162	12.275
3	DMAC	5.049	3377331	34.317	8.855
4	DMS	6.276	31290	0.318	10.549
Total			9841632	100.000	

Fig. 4: Solvent-screening chromatogram showing N, N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and N, N-dimethylacetamide (DMAc), the anisole peak formed by derivatisation of DMS under the optimised GC-HS-FID conditions

Analytical method validation

The developed GC-HS-FID method was validated following the ICH Q2(R1) guidelines [23] to confirm its reliability for the indirect quantification of DMS in clobazam API samples. As shown in table 3, the method demonstrated excellent precision, with a % RSD of 0.49% for six replicate injections of DMS at a concentration of 1.5 ppm using the DB-5 GC column. The validation process, as per ICH recommendations, included evaluation of system suitability, specificity, accuracy, limit of detection (LOD), limit of quantification (LOQ), range, linearity, precision, and solution stability. Statistical analysis was performed using Microsoft Excel, in which linearity was evaluated by least-squares regression, and precision and accuracy were expressed as % RSD.

Specificity

The specificity of the method was confirmed by comparing the chromatograms of the derivatised DMS standard, the unspiked clobazam API sample, and the blank diluent. The derivatised DMS showed a clear anisole peak at about 6.30 min, as seen in panel A of fig. 5. Panels B and C showed no peaks at the analyte retention window for the unspiked API or the blank diluent, demonstrating that neither the matrix nor the reagents introduced any interference.

The USP resolution between DMF and anisole (~6.3 min) was 10.99, confirming baseline separation and demonstrating that matrix components do not interfere with anisole (the derivatised form of DMS). The chromatographic performance further supported the method's selectivity, with a tailing factor of 1.706 and a theoretical plate count of 35,954 for the anisole peak.

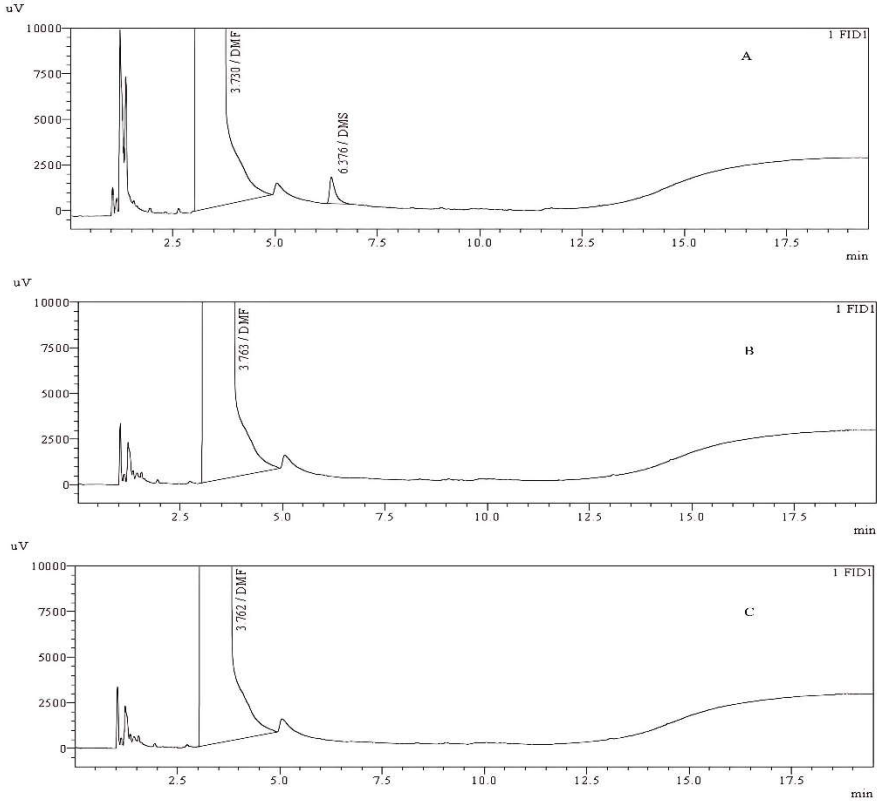


Fig. 5: Combined GC–HS–FID chromatograms demonstrating analyte detection and method specificity, (A) Derivatised dimethyl sulfate (DMS) showing a clear anisole peak at ~6.37 min. (B) Unspiked clobazam API sample with no peak at the anisole retention time. (C) Blank diluent showing no interfering signals near the analyte retention window

Precision (method and intermediate precision)

Method precision (repeatability) and intermediate precision of the developed GC–HS–FID method were evaluated in accordance with ICH Q2(R1) guidelines, and the results are presented below.

Method precision

Method precision, also known as repeatability, was evaluated by performing six replicate injections of a standard DMS solution under identical experimental conditions within a single analytical run (on the same day, with the same analyst and instrument). The RT and peak response for each injection were recorded, and the results are presented in table 3.

The peak responses for the six injections ranged from 14,235 to 14,413, with RT varying only slightly from 6.302 to 6.306 min. The mean peak response was 14,303, with a standard deviation of 70.04, yielding a % RSD of 0.49%, which is well within the generally accepted limit of ≤2.0% for analytical precision. The RT also showed minimal variation, with an RSD of 0.03%.

These results confirm that the method yields highly consistent outputs when applied under fixed parameters, thereby supporting its suitability for routine analysis.

Intermediate precision

Intermediate precision was evaluated by analysing six replicate injections of a standard dimethyl sulfate solution under varied conditions, including different days, analysts, columns, and instruments, and the results are presented below.

As shown in table 3, the RT for intermediate precision ranged from 6.330 to 6.333 min, and the peak responses ranged from 13,169 to 13,345. The mean peak response was 13,232, with a standard deviation of 60.84, yielding an RSD of 0.46%.

These low % RSD values for both the area response and RT under intermediate-precision testing demonstrated the robustness and reproducibility of the method across different operational scenarios, confirming that it can deliver consistent results even under typical variations expected in routine laboratory use.

Although the mean peak areas for method precision (14,303) and intermediate precision (13,232) differ by approximately 7 %, this systematic shift is attributable to routine differences in instrument/column response between runs (e. g., detector sensitivity, column age, minor gas-flow variation).

Importantly, within each experiment, the % RSD values remained ≤0.5%, demonstrating excellent repeatability under both conditions. In practice, a fresh calibration curve is generated for each analytical session, so such between-run differences in absolute response do not impact the accuracy of quantitation.

Precision and intermediate results are summarised in table 3.

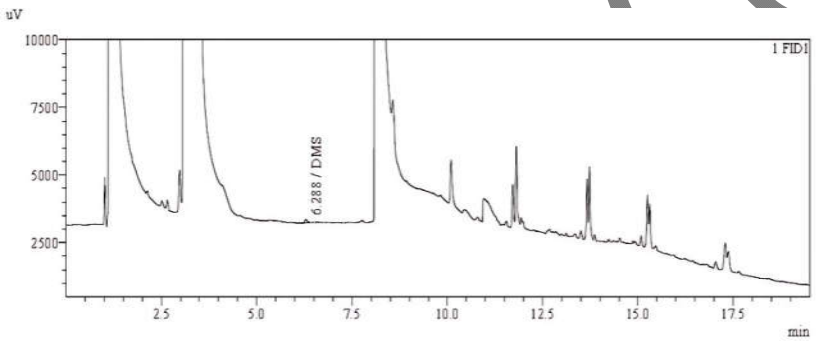
Table 3: Precision (method and intermediate precision)

Run no.	RT (min) – method precision	peak response – method precision	RT (min) – intermediate precision	peak response – intermediate precision
1	6.303	14362	6.331	13345
2	6.302	14413	6.330	13224
3	6.306	14281	6.330	13191
4	6.305	14235	6.331	13169
5	6.302	14244	6.333	13227
6	6.305	14284	6.333	13233
Mean	6.304	14303	6.331	13232
SD	0.002	70.04	0.001	60.84
% RSD	0.03	0.49	0.02	0.46

value represent n = 6. RT: retention time; SD: standard deviation; RSD: relative standard deviation.

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

The "LOD and LOQ" were established by evaluating the lowest concentrations of DMS that could be reliably detected and quantified within the sample matrix. Using the "signal-to-noise (S/N) ratio" approach, the quantitation limit was determined to be 0.12 ppm, with an average signal-to-noise ratio of 12.56, and the detection limit was set at 0.04 ppm, supported by an S/N ratio of 4.51. The results are summarised in table 4. Representative chromatograms of the LOD and LOQ levels are presented in fig. 6 and fig. 7.

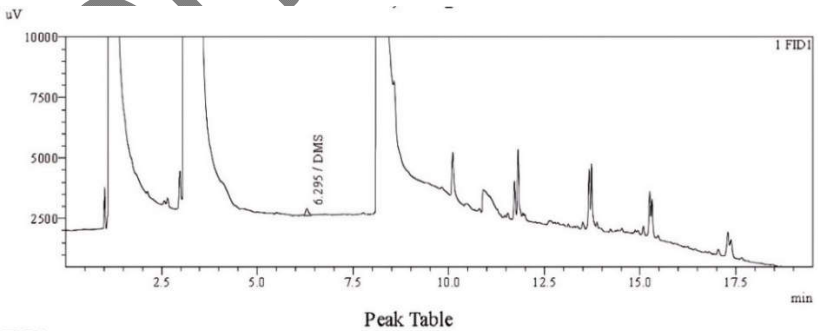


Peak Table

FID1

Peak#	Name	Ret. Time	Area	Area%	S/N
1	DMS	6.288	544	100.000	4.46
Total			544	100.000	

Fig. 6: LOD level chromatogram containing dimethyl sulfate (0.04 ppm)



Peak Table

FID1

Peak#	Name	Ret. Time	Area	Area%	S/N
1	DMS	6.295	1541	100.000	13.26
Total			1541	100.000	

Fig. 7: LOQ level chromatogram containing dimethyl sulfate (0.12 ppm)

Table 4: GC-HS-FID analysis of DMS: LOD and LOQ determination based on S/N ratio

S. No.	Anisole area – LOD (0.04 ppm)	S/N – LOD	RT (min) – LOD	Anisole area – LOQ (0.12 ppm)	S/N – LOQ	RT (min) – LOQ
1	544	4.46	6.28	1541	12.42	6.29
2	530	4.57	6.29	1536	12.00	6.29
3	545	4.50	6.29	1573	13.26	6.29

Average	540	4.51	6.29	1550	12.56	6.29
SD	8.39	0.06	0.01	20.07	0.64	0.00
% RSD	1.55%	1.23%	0.09%	1.30%	5.11%	0.00%

Value represent n = 3. LOD: limit of detection; LOQ: limit of quantification; RT: retention time; SD: standard deviation; RSD: relative standard deviation; S/N: signal to noise ratio

Linearity and range

As part of the method validation, the linearity and range of the developed GC-HS-FID method were assessed to verify its capability to generate accurate and proportional responses across the specified concentration range. The Standard solutions of anisole derivative (from DMS) were prepared at seven concentration levels corresponding to 8%, 25%, 50%, 80%, 100%, 120%, and 150% of the target working concentration (1.5 ppm). The prepared solutions were analysed by chromatographic techniques, and the resulting data were used to evaluate the method's linearity, as shown in table 5 and illustrated in fig. 8.

Table 5: Linearity of the DMS–anisole derivative response

DMS concentration (ppm)	% of working concentration (1.5 ppm)	Injection 1 (peak area)	Injection 2 (peak area)	Injection 3 (peak area)	Average (peak area)	% RSD
0.12	8	1585	1609	1602	1599	0.77 %
0.38	25	3589	3477	3433	3500	2.30 %
0.75	50	7138	7146	7152	7145	0.10 %
1.20	80	11333	11330	11312	11325	0.10 %
1.50	100	14279	14294	14282	14285	0.06 %
1.80	120	16939	16936	16958	16944	0.07%
2.25	150	20806	20815	20811	20811	0.02 %

value represent n = 3. RSD: relative standard deviation. Linearity level corresponds to the actual DMS concentration in solution; "% of target concentration (1.5 ppm)" indicates the proportion of the working level. Average values are mean peak areas from triplicate injections; % RSD is calculated from replicate areas.

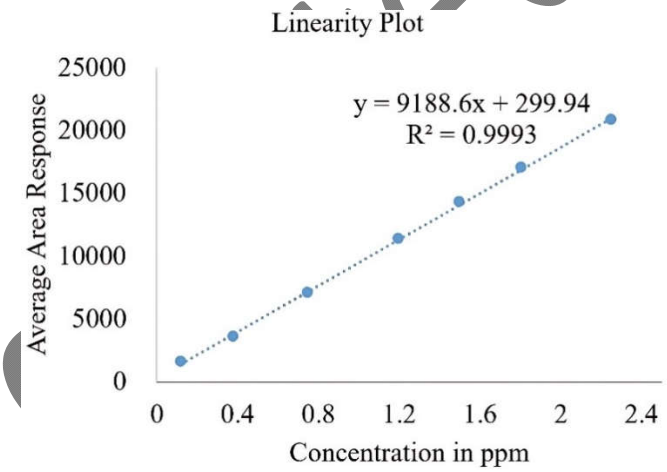


Fig. 8: Dimethyl sulfate derivative linearity plot: average peak area vs. concentration (ppm)

Table 6 presents the corresponding regression statistics. This evaluation confirms the suitability of the method for quantitative analysis within a specified concentration range.

Table 6: Linearity-based regression statistics

Parameter	Value
Slope (m)	9188.55
Intercept (c)	300.08
R^2	0.9993
Standard Error	106.95
p-value	4.05×10^{-9}

Regression based on average values; n = 3 injections per level. R^2: coefficient of determination.

In the regression analysis, the coefficient of determination (R^2) was 0.9993, indicating excellent linearity over the tested concentration range (0.12 to 2.25 ppm). The low standard error and highly significant p-value further confirm the model's statistical robustness and reliability. The slope of 9188.55 demonstrates high sensitivity, reflecting a strong signal response per part per million (ppm) of the analyte.

Accuracy

The method's accuracy was assessed by quantifying and recovering known analyte concentrations with high precision, confirming its reliability and suitability for real-world sample analysis. Three replicate sample solutions were prepared at concentrations of 25%, 50%, 100%, and 150% of the target concentration by spiking a homogeneous clobazam API matrix with known quantities of DMS. The spiked samples were analysed using the validated GC–HS method, and the recovery amounts were calculated to evaluate the accuracy of the method. The observed recoveries ranged from 99.83% to 100.14% across the tested concentration levels, demonstrating the method's accuracy. The detailed accuracy results are presented in table 7.

Table 7: Accuracy evaluation of DMS in clobazam across multiple spiked levels

Level (%)	Injected conc. (ppm)	Prep 1	Prep 2	Prep 3	Mean peak area	Conc. recovered (ppm)	% Recovery	SD	% RSD
25	0.380	3047	3062	3029	3046	0.376	100.14	16.52	0.54
50	0.750	6042	6061	6030	6044	0.751	100.09	15.63	0.26
100	1.500	12009	12012	12018	12013	1.497	99.83	4.58	0.04
150	2.250	18017	18052	18049	18039	2.251	100.06	19.40	0.11

value represent n = 3. SD: standard deviation; RSD: relative standard deviation; ppm: parts per million.

The low RSD values observed across all tested concentration levels did not exceed 0.54%, confirming the method's high repeatability and minimal response variability. Additionally, the method demonstrated strong linearity, as evidenced by a consistent and proportional increase in the average peak area with increasing concentration from 25% to 150%, validating its suitability for accurate quantification across a broad range. The low standard deviations further underscore the robustness of the method, particularly at the 100% concentration level, where the standard deviation is only 4.58, highlighting its stability and reliability under standard operating conditions.

Robustness

The robustness of the GC-HS-FID method was evaluated by examining its performance under deliberately varied analytical conditions and assessing its consistency in delivering reliable and

accurateresults despite minor changes in experimental parameters. To demonstrate the method's resilience and applicability for routine use, a series of experiments was conducted in which key gas chromatographic parameters were systematically modified to assess their impact on method stability and performance. Specifically, the carrier gas flow rate was varied between 2.92 and 3.56 mL/min, the detector temperature was adjusted to 235 °C to 245 °C, and the initial oven temperature was fine-tuned between 85 °C and 95 °C, as detailed in table 8. These controlled modifications were designed to simulate the typical variations that may occur during routine analysis. The method maintained consistent performance despite deliberate variations in the analytical conditions, confirming its robustness. Additionally, the peak area responses showed an RSD of less than 1%, confirming the robustness and reliability of the method for consistent quantitative analysis.

As expected for an FID-based method, detector temperature had a marked influence on absolute signal intensity, with mean anisole areas ranging from 8,647 at 235 °C to 12,375 at 245 °C (table 8). At the same time,the % RSD within each condition remained ≤0.5%. This behaviour reinforces the need for the method to always use relative quantification against a calibration curve generated under the same detector settings, rather than relying on absolute peak areas. When calibration is performed in each run, these temperature-dependent differences in response do not compromise the accuracy or precision of DMS quantitation.

Table 8: Robustness evaluation of the optimised GC-HS-FID method

Parameter	Optimised value	Varied value	mean area response	% RSD
flow rate	3.24 ml/min	2.92 ml/min	9475	0.10
flow rate	3.24 ml/min	3.56 ml/min	9632	0.50
oven temperature	90 °C	85 °C	10550	0.34
oven temperature	90 °C	95 °C	11616	0.18
detector temperature	240 °C	235 °C	8647	0.37
detector temperature	240 °C	245 °C	12375	0.19

value represent n = 6. RSD: relative standard deviation. The effects of deliberate variations in flow rate, oven temperature, and detector temperature on the mean area response and % RSD are presented to assess the method's reliability under minor operational changes.

Solution stability

The stability of DMS was assessed over 72 h at room temperature (25±2 °C) using GC-HS-FID with the samples stored in clear glass containers under ambient laboratory conditions.

Table 9: Solution stability results

Time interval	Mean area	% RSD	% Decrease from initial

0 h	13924	0.43	0.00
24 h	12572	0.23	9.70
48 h	11743	0.33	15.65
72 h	10645	0.24	23.57

value represent n = 6. RSD: relative standard deviation; h: hour

The method exhibited excellent precision, as indicated by % RSD values consistently below 0.5% at all time points. However, a progressive decline in the peak area was observed, with reductions of 9.7% at 24 h, 15.65% at 48 h, and 23.57% at 72 h relative to the initial measurement. The approximately 10 % loss observed after 24 h represents a notable limitation for quantitative work at trace levels and supports the recommendation that standards and samples be prepared freshly and analysed within 24 h. This trend indicates a time-dependent decline in the anisole response, consistent with degradation or volatilisation of derivatised DMS under test conditions. The corresponding solution stability data are listed in table 9. Although this method is robust and reproducible, the findings indicate that DMS exhibits limited solution stability at room temperature. Therefore, it is recommended that analytical solutions be utilised within 24 h of preparation to ensure accurate quantification.

Batch analysis

Three different batches of clobazam API samples were analysed using the developed and validated GC-HS-FID method for the indirect determination of DMS. This analysis was performed to evaluate the presence of DMS in routine production samples. DMS was not detected in any of the three batches, indicating that the levels were below the established LOD. Table 10 summarises the results.

Table 10: Batch analysis of dimethyl sulfate (DMS) in clobazam API by GC-HS-FID

Batch number	Dimethyl sulfate (ppm)
Batch 1	Below detection limit
Batch 2	Below detection limit
Batch 3	Below detection limit

"Below detection limit" with reference to the established LOD.

In silico prediction results for DMS

An in silico mutagenicity assessment of DMS using the VEGA in silico tool revealed consistent positive predictions across the three validated models. The CONSENSUS model predicted DMS to be mutagenic with a score of 1.0, reflecting agreement across multiple internal models. The CAESAR model also classified DMS as mutagenic with high reliability and within the applicability domain. The ISS model predicted a positive outcome and identified a structural alert (SA2) associated with the alkyl esters of sulfonic acids, although the compound was partially outside its applicability domain. The KNN-Read-Across model supported a mutagenic classification based on its high similarity to known mutagens within the domain. The detailed VEGA QSAR model predictions for DMS are provided in the Supplementary Material. The QSAR model predictions and the resulting classification are provided in table 11. Taken together, these predictions provide strong computational evidence that DMS is mutagenic, consistent with its known chemical reactivity, and an established classification as an alkylating agent. These findings support the classification of DMS as a Class 1 GTI under ICH M7 (R1)**,** which requires stringent control to limit patient exposure.

The isolated non-mutagenic prediction from SARpy-IRFMN was generated outside its applicability domain and lacks support from structural alerts or known experimental data for alkyl sulfates; consequently, it was considered unreliable in the context of the overall weight of evidence.

Table 11: In silico (Q)SAR predictions for the mutagenicity assessment of DMS as per ICH M7 (R1)

Model name	Endpoint	Prediction	Applicability domain (AD)	Reliability	ICH M7 classification
CONSENSUS (1.0.4)	Mutagenicity (Ames)	Mutagenic	in AD	strong (score 1.0)	Class 1 (positive)
CAESAR (2.1.14)	Mutagenicity (Ames)	Mutagenic	in AD	high, consistent with evidence	Class 1 (positive)
ISS (1.0.3)	Mutagenicity (Ames)	Mutagenic	partial AD	structural alert SA2	Class 1 (positive)
SARpy-IRFMN (1.0.8)	Mutagenicity (Ames)	Non-mutagenic	outside AD	low (domain limitation)	outside AD (not reliable)
KNNread-across (1.0.1)	Mutagenicity (Ames)	Mutagenic	in AD	high similarity to known mutagens	Class 1 (positive)

AD, applicability domain

Expert review of DMS under ICH M7 guidelines

An expert review of DMS was conducted in accordance with ICH M7 (R1) guidelines to assess its mutagenic potential. The chemical structure of DMS (SMILES: O=S(=O)(OC)OC) contains two electrophilic methyl groups that are capable of alkylating nucleophilic sites in biomolecules, particularly DNA. Mechanistically, DMS methylates DNA bases, leading to the formation of mutagenic adducts, such as O6-methylguanine, which can cause mispairing and point mutations. These features are characteristic of direct-acting, DNA-reactive genotoxic compounds.

To support this mechanistic understanding, a panel of in silico (Q)SAR models implemented on the VEGA platform was used to predict mutagenicity. The models included CONSENSUS, CAESAR, ISS, SarPy-IRFMN, and KNN-Read-Across. Each prediction was assessed for its applicability domain (AD), reliability, and presence of structural alerts. The majority of models, particularly CAESAR, ISS, and KNN, predicted DMS to be mutagenic, within their

applicability domains. And supported by known structural alerts, such as alkyl esters of sulfonic acids. The only discordant model, SARpy-IRFMN, fell outside its applicability domain and was not considered in the weight-of-evidence assessment.

In line with ICH M7 Section 2.3.2, expert judgment was applied to interpret the overall weight of the evidence. Concordant positive predictions from at least two complementary models within their applicability domains were deemed sufficient to classify DMS as mutagenic. Considering its established alkylating reactivity and known biological mechanism, DMS was concluded to be a Class 1 GTI under ICH M7, requiring strict regulatory control and analytical monitoring to limit patient exposure.

DISCUSSION

As summarised in table 1, most previously reported analytical methods for dimethyl sulfate rely on GC–MS or LC–MS techniques and typically achieve limits of detection at or above the low-ppm level with longer analytical runtimes, whereas the present GC–HS–FID method provides sub-ppm sensitivity with a shorter cycle time, making it more suitable for routine quality-control applications. The analytical and toxicological assessment performed in this study provides an integrated evaluation of dimethyl sulfate (DMS) as a high-risk process-related impurity in clobazam API. The in silico (Q)SAR assessment using multiple VEGA models consistently classified DMS as mutagenic within their applicability domains, supporting its classification as a Class 1 genotoxic impurity under ICH M7(R1). While the genotoxicity of DMS is well established, inclusion of a transparent and guideline-compliant QSAR assessment strengthens the regulatory justification [23, 24] and supports the requirement for highly sensitive analytical control.

Analytical determination of volatile and highly reactive genotoxic impurities such as DMS remains challenging due to their instability, lack of chromophores, and occurrence at trace levels. Recent studies have highlighted the suitability of derivatisation-based and headspace GC techniques for reliable quantification of such impurities, particularly when present at sub-ppm levels [25]. The GC–HS–FID method developed in this work addresses these challenges by combining efficient derivatisation of DMS to anisole with optimised chromatographic and headspace conditions, enabling stable detection and reproducible response.

Several GC-based methods have been reported for electrophilic alkylating impurities in pharmaceutical matrices, including GC–EI–MS determination of methyl-4-chlorobutyrate in moxifloxacin hydrochloride [26], supporting the general suitability of GC techniques for low-level monitoring of genotoxic impurities. In comparison with a previously reported GC–HS method for DMS determination in methoxsalen API [19], which employed phenol–NaOH derivatisation, DMSO as diluent, a wide-bore DB-5 column, and a 46 min analytical cycle with LOD and LOQ of 2.0 ppm and 5.0 ppm, respectively, the present method demonstrates markedly improved analytical performance.

The use of potassium hydroxide and a DMF–water system in the present method resulted in improved derivatisation efficiency and sharper chromatographic peaks, while the narrower DB-5 column enabled a reduced GC runtime of 28 min without compromising resolution. The achieved limit of detection (0.04 ppm) and limit of quantification (0.12 ppm) provide a substantial sensitivity advantage, ensuring reliable monitoring of DMS well below the regulatory threshold required for clobazam API.

From a practical perspective, the use of GC–HS–FID offers advantages for routine quality-control laboratories compared with mass spectrometric techniques, including lower operational cost, reduced maintenance requirements, and sufficient sensitivity for ICH M7-compliant control of DMS. The method demonstrated excellent specificity, linearity, precision, and accuracy, with no interference observed at the anisole retention time, and no detectable DMS was observed in the commercial clobazam API batches analysed.

Overall, the developed GC–HS–FID method provides a robust, sensitive, and regulatory-aligned analytical approach for routine monitoring of dimethyl sulfate in clobazam API and may be readily applicable to other APIs synthesised using DMS as a methylating agent.

CONCLUSION

In silico (Q)SAR analysis confirmed the mutagenic potential of DMS, supporting its classification as a Class 1 GTI under ICH M7 and underscoring the need for stringent analytical control. A GC–HS–FID method was developed and validated for the trace-level determination of DMS in clobazam API, using an indirect derivatisation approach to enable reliable detection. The method is suitable for routine quality-control monitoring of DMS in clobazam and other APIs synthesised using DMS as a methylating agent.

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AUTHORSHIP CONTRIBUTION

Srinivas Birudukota-Conceptualisation, Methodology, Data Curation, Formal Analysis, Investigation, Validation, Visualisation, Writing Original Draft, Review and Editing. Dr Swagata Halder and Dr Ramesha Andagar Ramakrishna: Data curation, writing review and editing, and supervision.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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