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REPEATED PRESSURE-RELATED CHROMATOGRAPHIC FAILURE DURING SALIVARY CORTISOL ANALYSIS BY LC-HRMS/MS: A LABORATORY CASE REPORT AND HYPOTHESIS-DRIVEN ROOT CAUSE ASSESSMENT

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Abstract *Liquid chromatography tandem mass spectrometry (LC-MS/MS) is the reference standard method for quantitatively measuring salivary cortisol, largely because of its high analytical specificity and sensitivity. However, saliva is a complex biological matrix that may compromise chromatographic robustness during routine testing. Here, we report a laboratory case of system back-pressure exceedance, chromatographic deterioration, and analytical column collapse during routine LC-HRMS/MS analysis of salivary cortisol using a commercially available sample preparation and analysis kit. Two columns used in assay analysis failed consecutively. Both columns were subjected to the same working conditions. Factors such as how the samples were injected, chromatograms, and the behaviour of the system were analyzed. Possible causes of failures were also evaluated based on the pressure and behaviour of the columns. The team also evaluated possible causes based on column pressure and behaviour. The review indicated that the matrix caused the failure. Poorly removed salivary mucins and other particles clogged the columns. Flow restriction caused retention time to vary. The columns became worn out because the phase failed to remain permanently fixed to the column. Failures occurred in two columns and suggested a limitation of the method regarding matrix interaction with LC-MS/MS. This occurred mainly when high-viscosity saliva samples were analyzed using UHPLC columns. Restrictions were noted particularly at the column inlet. It was recommended that columns be well protected and that preventive maintenance be performed to reduce column failures and loss of phase integrity. This is especially true in laboratories that analyze salivary cortisol using LC-MS/MS. The report presents a case study illustrating the types of column failures caused by matrix limitations in LC-MS/MS analysis.*

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Keywords*:* *LC-HRMS/MS; Orbitrap; PRM; salivary cortisol; column fouling; back-pressure; matrix effects; method robustness; sample preparation; root-cause analysis*

1. Introduction

Salivary cortisol is an increasingly common non-invasive measure of HPA axis function in clinical and research settings. It is a free-fraction measure, is not affected by cortisol-binding globulin, and offers particular advantage for assessing the diurnal cortisol rhythm and the stress response, as well as for screening for Cushing's syndrome (Raff, 2009; Mezzullo et al., 2016). Measuring the free fraction of cortisol can be challenging because residual food, blood, and topical corticosteroids can contaminate the sample before collection. LC-MS/MS is considered the reference method for steroid hormone analysis because it offers higher analytical specificity and sensitivity than immunoassays (Keevil, 2016; Vogeser and Seger 2008). However, saliva as an analytical matrix presents challenges because variability in viscosity, protein content, mucin burden, and particulates can alter analyte retention over time (Taylor, 2005; Chiappin et al., 2007). Further, short-term validation studies cannot always capture significant alterations in a method's long-term matrix performance (Matuszewski et al., 20023; Van Eeckhaut et al., 2009). The bioanalytical LC-MS/MS literature also describes how using sub-2 µm UHPLC columns with un-purified biological matrices can lead to rapid column fouling and failure (Rodríguez-Cabo et al., 2009; Zuvela et al., 2019). Column failure in a routine clinical laboratory has significant economic and operational consequences. Although column failure is a known risk, relatively few documented cases of complete failure have been reported with commercially available salivary cortisol assay kits under routine conditions.

Studies like this are crucial because they highlight the gap between controlled method-validation findings and what happens in a working lab, and because they

give other laboratories a practical framework for addressing the same problems. Here we describe a reproducible pattern of back-pressure exceedance and analytical column failure that we encountered during routine LC-HRMS/MS analysis of salivary cortisol in an ISO 15189-accredited clinical laboratory. Here, we describe a repeatable pattern of excessive back-pressure and analytical column failure that occurred during routine LC-HRMS/MS testing of salivary cortisol in our ISO 15189-accredited clinical laboratory. This report walks through what we observed when the failures occurred, why we think they happened based on sound chromatographic reasoning, and what laboratories can do to manage them. We see this as a matrix-method interaction issue rather than a fault of any specific product, and we hope it helps anyone considering or already running LC-MS/MS salivary cortisol testing.

2. Materials and Methods

2.1 Setting

Analyses were performed in the clinical mass spectrometry section of LifeCore Private Clinic, Abu Dhabi, UAE - an ISO 15189-accredited clinical laboratory. Salivary cortisol testing was performed on routine patient specimens referred for HPA axis function assessment.

2.2 Instrumentation

Analyses were performed using a Thermo Scientific Q Exactive Plus hybrid quadrupole Orbitrap high-resolution mass spectrometer coupled to a UHPLC system (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization (HESI-II) source operated in positive ion mode. Chromatographic separation was coupled online to high-resolution mass spectrometric detection. Data acquisition and processing were performed using Xcalibur software (Thermo Fisher Scientific).

2.3 Chromatographic Conditions

Chromatographic separation was achieved on a reversed-phase analytical column Poroshell 120 EC-C18 (50 × 2.1 mm, 1.9 µm). The mobile phase consisted of Mobile Phase M1 (Pump A) and Mobile Phase M2 (Pump B), supplied with the kit. Delivered under gradient conditions as specified by the kit manufacturer. The flow rate was 0.400 mL/min, the column temperature was 45°C, and the injection volume was 15 µL. The total run time per injection was 7.50 min.

2.4 Mass Spectrometric Conditions

Quantification was performed in parallel reaction monitoring (PRM) mode using the transitions m/z 363.2 → 121.1 (cortisol, quantifier) and m/z 363.2 → 97.0 (cortisol, qualifier). The deuterated internal standard cortisol-d4 was monitored at m/z 367.2 → 121.1. Mass spectrometric source parameters were optimised to maximize signal response for low-level standard solutions.

2.5 Sample Collection and Pre-analytical Handling

Saliva samples were collected using Salivette® Cortisol or equivalent saliva collection devices. Subjects were instructed to remain fasting and avoid intense physical activity for at least 2 h before sampling. Ten minutes before collection, participants rinsed their mouths with cold water, and we recorded the collection time. Samples were centrifuged immediately after collection to improve stability, and the clarified saliva was subsequently processed for LC-HRMS/MS analysis according to the manufacturer's protocol.

2.6 Sample Preparation

Sample preparation was performed using the Eureka Cortisol and Cortisone in Saliva by LC-MS/MS kit (REF LC91110; Eureka S.r.l., Lab Division, Italy) according to the manufacturer's instructions. Briefly, transfer 500 µL of saliva sample into an Eppendorf tube and mix with 10 µL of Internal Standard Solution (Reagent A) and 20 µL of Clarifying Solution (Reagent B). Vortexed the mixture for 20 seconds, then centrifuged at 14,000 rpm for 10 minutes. Transferred a 300 µL aliquot of the supernatant to a fresh Eppendorf tube, combined it with 100 µL of Stabilization Solution (Reagent C), and vortexed for an additional 20 seconds. The extract was then kept in an autosampler vial and injected at 15 µL for LC-HRMS/MS analysis.

2.7 System Suitability and Performance Monitoring

System back pressure was monitored during analytical runs to ensure it stayed within both the manufacturer's specified and the method-defined limits.

2.8 Column History

Two different analytical columns were used during the investigation. Each column was dedicated for the analysis of salivary cortisol. Column A was installed on 08 September 2025 and expired after ~15 injections. Column B was installed on 16 October 2025 and also failed after ~15 injections. Columns A and B were brand new when installed, and we ran them under the manufacturer's recommended conditions for pressure, temperature, and mobile phase.

3. Results

3.1 Increased Backpressure

Both the columns used for this analysis (A and B) started having problems after ~10 injections. Problems with Column A (installed 08 September 2025) started when the instrument raised communication errors between the pumps (Figure 1). Problems with Column B (installed 16 October 2025) began with leak detection alarms (Codes 33 and 34) and eventually led to a method abort (Figure 2). Since we observed the same issues with two different columns, we believed the issue was with the LC-MS/MS system and its associated hardware.



		Date	Time	Retention Time	Device	Message
1		08/09/2025	1:19:30 PM +04:00		SamplerModule	Driver has disconnected.
2		08/09/2025	1:19:20 PM +04:00		ColumnComp	Driver has disconnected.
3		08/09/2025	1:19:20 PM +04:00		PumpModule	Driver has disconnected.
4		08/09/2025	1:19:19 PM +04:00		PumpModule	Error reading from VPUMP @ USB-0315592. A device attached to the system is not functioning.
5		08/09/2025	1:19:19 PM +04:00		SamplerModule	Error reading from VAS @ USB-0315639. A device attached to the system is not functioning.
6		08/09/2025	1:19:19 PM +04:00		ColumnComp	Error reading from VTCC @ USB-6504426. A device attached to the system is not functioning.
7		08/09/2025	10:25:24 AM +04:00	-1.395		Running sequence queue intercepted by user Sampathkumar.
8		08/09/2025	10:02:52 AM +04:00		SamplerModule	Temperature control is on but not all doors are closed. Close both doors or temperature control will shut down in 20.0 minutes. Code 9032.
9		08/09/2025	10:01:40 AM +04:00		SamplerModule	Temperature control is on but not all doors are closed. Close both doors or temperature control will shut down in 20.0 minutes. Code 9032.

Figure 1. Communication error event (08 September 2025)

1738		16/10/2025	9:33:33 AM +04:00			Instrument method aborted (because an abort or fatal error has occurred).
1739		16/10/2025	9:33:33 AM +04:00	2.831	SamplerModule	Leak detected. Code 34.
1740		16/10/2025	9:32:53 AM +04:00	2.164	SamplerModule	Leak detected - eliminate within approx. 30 seconds. Code 33.
1741		16/10/2025	9:32:23 AM +04:00	1.664	SamplerModule	Leak detected - eliminate within approx. 60 seconds. Code 33.

Figure 2. Leak detection event (16 October 2025)

3.2 Clarification of Pump Error Messages

During the failure event, the pump recorded two pressure-related error messages. The first indicated that the maximum pressure limit had been reached and triggered an automated system shutdown. Shortly after, the second message, indicating a low pressure condition or leak, was generated. The second message likely resulted from the pressure drop after the system shut down. Figure 2 illustrates the sequence of events.

3.3 Chromatographic Deterioration

Analysis of the cortisol and internal standard chromatograms indicated deterioration in peak shape during analysis. The peaks became broad and lost symmetry, with increased baseline disturbance. Compared with the expected chromatographic profile, both the cortisol peak and the internal standard cortisol-d4 peak showed increased retention times, eluting at approximately 3.49 and 3.70 minutes, respectively. These results are consistent with decreased column performance and increased backpressure.

3.4 Internal Standard and Quantitative Performance

The retained internal standard (cortisol-d4) and the cortisol analyte peak indicated retention time drift. The retention time drift was due to a chromatographic issue, not mass calibration drift. Internal quality control values indicated increased imprecision and a negative bias at low values during the final stages before column failure. These values, along with the other observed deteriorations in chromatographic performance and column integrity, indicated failure of the chromatographic system.

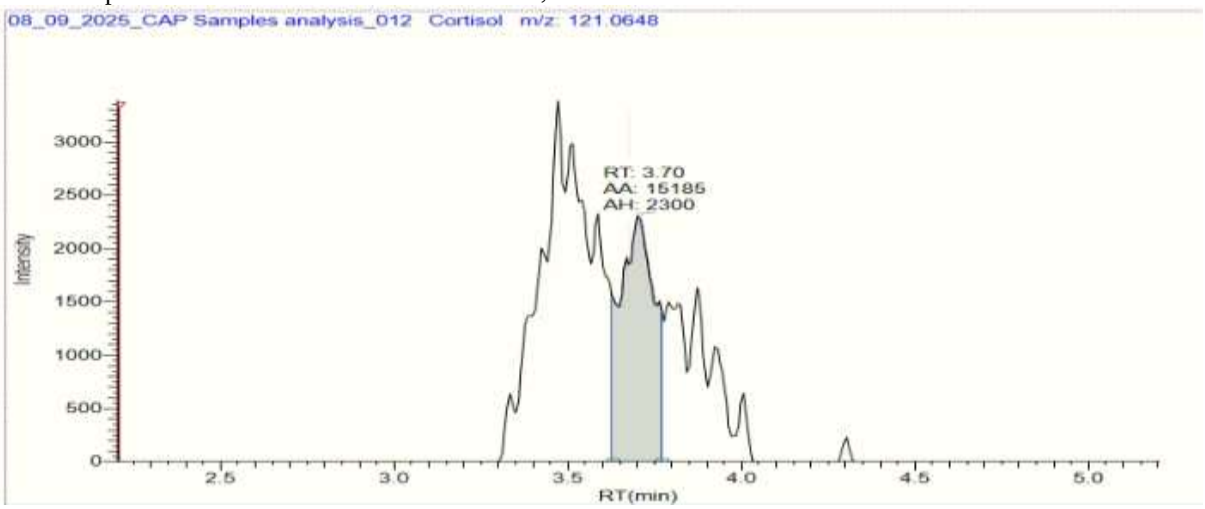


Figure 3 Representative chromatograms of Cortisol

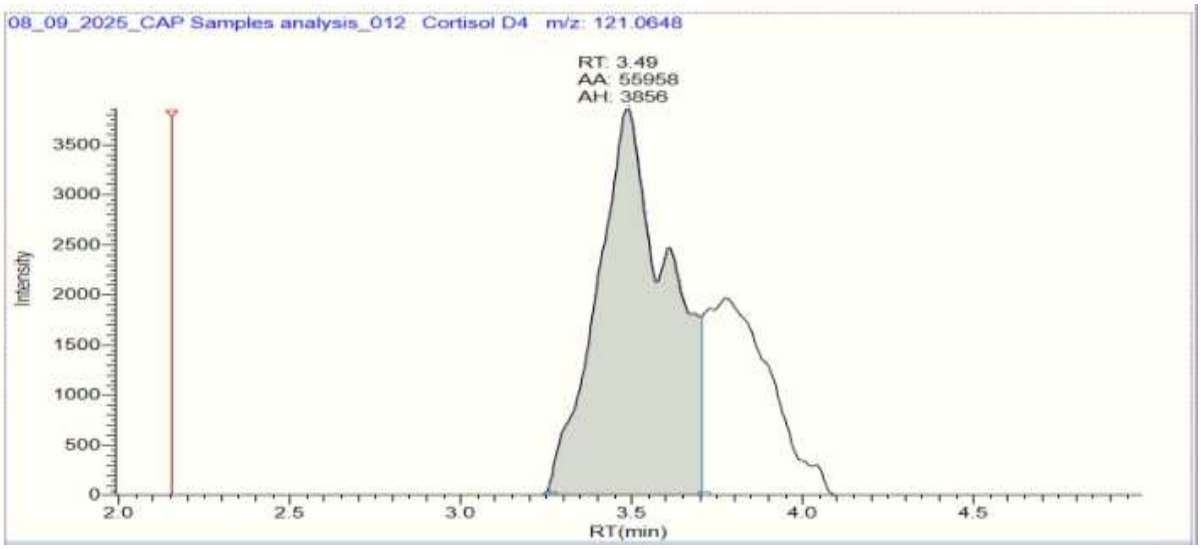


Figure 4 Representative chromatograms of Cortisol-D4

3.5 Postmortem Column Inspection

No post-mortem visual or microscopic examination, column disassembly, back-flushing experiments, surface characterization, or chemical analysis of retained samples was performed. As a result, the position and nature of the flow-restrictive materials could not be definitively established.

3.6 Impact on Operations

Failure of two analytical columns during the study period disrupted routine laboratory operations and required replacement of the failed columns. Analytical testing was delayed while corrective actions were being implemented. No patient data was released as a result of the instrument errors. Samples associated with the interrupted analytical sequence were re-analyzed after stable assay performance and system suitability were restored.

4. Root Cause Analysis

A root cause analysis was performed to understand potential failure mechanisms based on the available evidence. Each hypothesis was assessed by reviewing chromatographic data, column inspection findings, and the relevant analytical chemistry literature. The results are summarised in Table 1.

Table 1. Hypothesis-driven root-cause analysis of the observed failure pattern

Hypothesis	Expected evidence if true	Observed evidence	Assessment
Isolated column defect	Failure confined to a single analytical column, with subsequent columns operating normally under comparable conditions.	A similar pressure-related and chromatographic failure pattern occurred with two separate, newly installed assay-dedicated columns operated using the same analytical workflow.	**Less consistent with the observations** Recurrence with two columns makes an isolated single-column defect less likely; however, it was not formally excluded experimentally.
General instrument malfunction, including pump, injector, or seals	Comparable pressure abnormalities or diagnostic faults occurring independently of the salivary cortisol assay and potentially affecting other analytical methods on the same instrument.	Pressure-related interruption occurred during the salivary cortisol workflow, whereas other assays performed on the same instrument did not show a comparable recurrent pattern.	**Less consistent with the observations but not formally excluded** The assay-associated recurrence does not strongly support a generalized instrument malfunction.
Matrix-associated flow restriction	Deterioration in pressure and/or chromatographic performance during repeated injections of prepared biological-matrix extracts, with recurrence under comparable analytical conditions.	Repeated pressure-related interruptions and chromatographic deterioration occurred during sequential analysis of prepared saliva extracts with two analytical columns. However, the location and composition of any restriction were not directly determined.	**Plausible and most consistent with the observed pattern; not directly confirmed** Matrix-associated flow restriction is considered a working hypothesis rather than an established mechanism.

Incomplete removal of matrix constituents during sample preparation	Detectable residual protein, mucin, particulate material, or other matrix components after clarification, with reduced matrix burden and improved chromatographic robustness following additional clean-up.	The manufacturer-specified clarification procedure was used without an additional filtration or SPE step. Residual protein, mucin, or particulate burden was not measured, and no controlled comparison with enhanced sample preparation was performed.	**Potential contributing factor; not experimentally demonstrated.** Additional matrix removal requires prospective evaluation.
Column format/particle size contributing to susceptibility to flow restriction	Increased susceptibility to additional hydraulic resistance during repeated analysis of complex biological extracts, with improved robustness demonstrated using an alternative column configuration or protective device.	Both affected analytical columns used 1.9 µm particles. No controlled comparison with alternative particle sizes, column formats, guard columns, or in-line filters was performed.	**Possible contributing factor; not independently established.** The available data do not demonstrate that the sub-2 µm column format itself caused the failure.

Taken together, what we saw best fits a sample matrix that gradually increased resistance to flow through the system as prepared saliva extracts were injected repeatedly. Two things may have played a part: matrix components that were not fully removed, and the use of sub-2 µm columns. That said, we never pinpointed where the restriction was located, and we did not characterize any material that might have been retained. For that reason, we treat both explanations as working hypotheses, not as causes we have confirmed experimentally.

5. Discussion

LC-MS/MS is the most common technique for quantifying steroid hormones. This technique uses isotopically labeled internal standards for quantification, offers high specificity, and shows minimal cross-reactivity (Keevil, 2016; Vogeser and Seger 2008), making it attractive for adoption in clinical laboratories. This case illustrates that, in day-to-day analysis, systematic variations introduced by biological matrices may affect chromatography more than method validation studies performed in a controlled lab environment. Of all biological matrices, saliva presents the greatest challenge because of its viscosity, protein content, mucins, cells, and particulate matter (Taylor, 2005; Chiappin et al., 2007). Leaving behind matrix constituents during sample preparation may adversely affect chromatography. In this work, however, we did not directly assess the burden of protein, mucin, and particulate matter and therefore should consider it a possible factor, not a demonstrated one. Retention time drift and peak broadening are consistent with increased flow resistance in the chromatographic system. However, because we did not perform other possible failure-mode checks (e.g., physically examining and back-flushing the columns), the location and nature of the failure remain unknown. So, for now, we see matrix-related flow restriction as a reasonable working hypothesis, not a confirmed mechanism.

Our sample-preparation workflow stopped at the manufacturer-specified clarification procedure, with no additional filtration or solid-phase extraction (SPE) afterward. Other ways to remove more matrix, such as a second clarification, filtration, higher centrifugal force, or SPE, could be worth testing in future studies. That said, we did not compare any of these approaches in a controlled way here, so our data cannot tell us whether they would have prevented the pressure-related failures we saw. Also, any change to the commercial sample-preparation procedure would need proper analytical verification before being adopted.

5.1 Implications for Method Validation

These observations reinforce the importance of extended robustness testing as part of method validation for LC-MS/MS assays on viscous biological matrices. Standard validation parameters (accuracy, precision, linearity, recovery, lower limit of quantification) are necessary but not sufficient. We recommend that validation studies for saliva-based LC-MS/MS methods also include: (a) extended sequence testing over at least 200-300 injections of authentic clinical samples; (b) continuous back-pressure monitoring with pre-defined trend thresholds; (c) retention-time stability assessment across the same extended sequence; and (d) explicit evaluation of the effect of additional clean-up steps (filtration, SPE) on column life, separately from their effect on accuracy and precision.

5.2 Implications for Routine Operation

For laboratories already running salivary LC-MS/MS assays, we recommend proactively monitoring system back-pressure as a leading indicator of column health, similar to how internal quality control monitors analytical accuracy. If back-pressure continues to increase and reaches around 120–150% of baseline over a series of injections, it may indicate the column needs attention. Washing the column or replacing the guard column at this stage can help prevent a complete failure. Also monitor retention-time shifts, changes in peak shape, and lower signal-to-noise ratios, as they can indicate developing problems before they become more serious.

6. Mitigation and Practical Recommendations

Based on the observations and root-cause analysis, we recommend the following practices for laboratories operating or implementing LC-MS/MS assays on saliva or other viscous biological matrices:

Enhanced sample preparation. Add an extra filtration step to the kit's standard protocol to remove any remaining particles. One simple option is to filter the sample through a 0.22 µm syringe filter or a 96-well filter plate after protein precipitation. If you need to remove more matrix components, you can use mixed-mode solid-phase extraction (SPE), although it adds steps and complicates the overall procedure.

Column protection. Use an in-line guard column with the same chemistry as the analytical column. Guard columns are significantly less expensive than analytical columns and can be replaced on a pressure-trend-driven schedule, protecting the analytical column from most of the matrix load. Control of injection parameters. Adjust injection volume to a smaller value, if possible, while maintaining the assay's required sensitivity. Evaluate a two-fold dilution strategy, in combination of a larger volume injection of the extract, for high matrix samples. This can help reduce the absolute matrix mass per injection while maintaining the required assay sensitivity. Preventive maintenance and performance monitoring. Carry out routine column washes using a solvent sequence at defined intervals. Continuously monitor back pressure and retention times and define threshold action levels (e.g. +30% baseline). Local method verification. Thoroughly evaluate vendor validation data. If vendors do not validate saliva as a test matrix, perform a comprehensive evaluation using a defined sample set representative of the assay's routine workload.

7. Limitations

This study has some limitations that should be considered when interpreting the findings. First, the observations were made during routine clinical use rather than under controlled experimental conditions. Details such as the number of injections before column failure and the rate at which back-pressure increased were obtained from instrument logs after the events occurred, rather than from a predefined study protocol. Second, the commercial kit contains proprietary components, so the authors could not fully describe the sample-preparation chemistry. Finally, the column was examined after failure using visual inspection and back-flush testing only. More detailed investigations, such as electron microscopy or surface chemical analysis, were not carried out and could have provided further insight into the causes of column deterioration. Fourth, the report is based on experience at a single laboratory, and the generalisability of the findings to other instrument platforms or other saliva collection devices requires confirmation by other centres. Finally, a formal controlled comparison with and without additional filtration was not undertaken prospectively; the recommendation in favour of additional clean-up is based on mechanistic reasoning and the published literature rather than on a direct experimental comparison in this laboratory.

8. Conclusion

Progressive back-pressure exceedance and sequential column failure during routine LC-HRMS/MS analysis of salivary cortisol were observed with a commercial analytical kit using a protein-precipitation sample-preparation workflow. Our root-cause analysis points to an interaction between the sample matrix and the method. When particulates and proteins are not removed thoroughly enough, they gradually build up and foul the inlet of sub-2 µm UHPLC columns. We offer this report as a practical example of how far controlled method validation can fall short of the day-to-day robustness a method needs in real laboratory use, and as a framework for other laboratories facing similar problems. Our main recommendations are to validate methods with the matrix in mind, proactively monitor back-pressure, and add sample clean-up steps beyond those included in the kit workflow.

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Statements and Declarations

Ethics Approval

This report describes laboratory analytical performance and equipment failure. No patient-identifiable data are presented. Institutional ethics approval was not required for this analytical case report under institutional policy.

Data Availability

The corresponding author can provide the data upon reasonable request, subject to patient confidentiality constraints.

Author Contributions

SRU conceived the investigation, led the root-cause analysis, and drafted the manuscript. SE, MST, MRQ, SHSM, MMA, FA, FUA, and MMK contributed to the analytical investigation, data review, and critical revision of the manuscript. All authors approved the final version.

Declaration of Generative AI and AI-assisted Technologies in the Writing Process

During the preparation of this work, the author(s) used ChatGPT (OpenAI) to assist with language refinement and restructuring for clarity. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Conflicts of Interest

The authors confirm that they have no conflicts of interest.

Consent for publication

All the authors agree to publish the manuscript.

Acknowledgment

Not applicable.

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