doi.org/10.36721/PJPS.2026.39.8.235.1

Stability of serum cytokeratin 18-M30 under different storage conditions for drug-induced liver injury assessment

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Abstract: **Background**: CK18-M30 is an emerging biomarker to be used in patients who have liver damage from drugs or other causes and is now commonly used as a marker of liver apoptosis in patients with drug-induced liver damage. **Objectives**: To investigate the effects of different storage conditions on the stability of human cytokeratin 18-M30 (CK18-M30) in serum, this study aims to provide data-driven evidence on sample stability for deferred laboratory testing of clinical specimens that cannot be analysed immediately. **Methods**: A total of 22 serum samples from individuals undergoing routine physical examination were collected and had different concentration levels of CK18-M30 in the Fourth Affiliated Hospital, Zhejiang University School. Baseline value from the immediate test. The remaining samples were distributed into five groups. **Results**: Aliquots stored at -20°C in cryotubes for 3 days and 2-8°C separation gel tube samples for 3 days had >80% concordance with the baseline and were not different ($P > 0.05$). Conversely, 2-8°C cryotube aliquots stored for 3 or 7 days, as well as 2-8°C separation gel tubes stored for 7 days, had concordance rates below 80%, significantly discrepant from the baseline ($P < 0.01$). **Conclusions**: CK18-M30 is relatively stable at -20°C. Between 2 and 8°C, the separation gel tubes were more stable than the aliquot tubes for up to three days. Aliquot tubes stored at 2-8ºC were suboptimal, as the CK18-M30 became unstable after 3 days. The demonstrated stability characteristics of serum CK18-M30 are crucial for ensuring trustworthy biomarker-based safety evaluation when immediate analysis is not possible, given its expanding use as a safety and pharmacodynamic biomarker in pharmaceutical research, especially for tracking drug-induced liver injury in clinical trials and pharmacovigilance programs.

Keywords: Clinical trials; Cytokeratin 18-M30; Drug-induced liver injury; Pharmaceutical biomarker; Sample stability

Submitted on 13-01-2026 – Revised on 12-02-2026 – Accepted on 23-02-2026

INTRODUCTION

Keratins, an epithelial-specific subgroup of intermediate filament proteins, comprise more than 20 distinct polypeptide types and serve as the main components of the cytoplasmic cytoskeleton (Baktikulova *et al.*, 2026). In the liver, the primary function of keratins is to protect hepatocytes from apoptosis and necrosis. During hepatocyte apoptosis, keratin is cleaved by caspases at a conserved aspartic acid site, generating a fragment known as CK18-M30. The level of CK18-M30 has been used as a diagnostic and prognostic biomarker for acute and chronic liver diseases, and it correlates with histological inflammatory activity (Erkisa *et al.*, 2021). Serum or plasma levels are widely used better to define cell death in various forms of liver disease (Cebi *et al*., 2024). Serum cytokeratin 18-M30 has become a significant biomarker in the pharmaceutical sciences for assessing hepatocellular apoptosis associated with medication exposure (Fujihara., 2025). It is being used more and more in clinical trials, pharmacovigilance initiatives and drug development programs to help with the early identification and tracking of drug-induced liver damage. Due to logistical limitations, serum samples are frequently gathered from various locations and examined after varying storage times in these pharmacological contexts. Therefore, it is essential to understand the stability of CK18-M30 under various pre-analytical storage conditions to preserve analytical accuracy and ensure accurate biomarker data interpretation in medication safety evaluations (Hu *et al*., 2025; Zhang *et al.*, 2023).

Therefore, the primary aim of this study was to systematically evaluate the short-term stability of serum CK18-M30 under different storage temperatures, durations and tube types commonly encountered in clinical laboratory practice. Proper pre-analytical sample handling, such as specimen collection, processing and storage are very important. To date, however, none of them has been studied with respect to their stability. The stability of other keratin fragments has been tested in only a few studies; CYFRA 21.1 has been shown to remain stable in serum for 7 days at 4°C in this study (Canki *et al.*, 2024; Singh *et al*., 2023). According to the reagent manual, serum CK18-M30 is stable for up to 24 hours at 2-8°C and for 1 month at -20°C (Goralska *et al*., 2023). However, due to the limitations of laboratory testing, conducting tests frequently is time-consuming and inconvenient, as they require freezing. It is important to study the longest possible stable period for the sample in different storage conditions. In the current research, we should choose samples stored at 2-8°C for the exam.

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The goal of this study is to determine how consistent the serum CK18-M30 test results are across different sample tubes, storage duration, and storage temperature.

MATERIALS AND METHODS

A total of 22 serum samples with varying CK18-M30 concentrations were randomly collected from individuals undergoing routine physical examination with no documented acute or chronic liver disease at The Fourth Affiliated Hospital, Zhejiang University School of Medicine in 2025. The hospital's institutional review board approved all procedures.

All selected venous blood specimens were centrifuged at 2,000g for 10 min. An aliquot of serum was immediately assayed (within 1 hr post - centrifugation) to determine baseline CK18-M30 concentrations. All baseline measurements were performed within a single analytical run to minimize inter-run variability. Stored samples were analyzed in subsequent runs, using the same analyzer, identical calibration settings, and reagents from a single lot. Quality control materials were included in each run to ensure analytical consistency, and baseline samples were not re-measured alongside stored samples. The remaining serum was evenly divided into five storage groups (300 ul each): Group A: Stored at -20°C for 3 days in polypropylene aliquot tubes. Group B: Stored at 2–8°C for 3 days in polypropylene aliquot tubes. Group C: Stored at 2–8°C for 3 days in inert separation gel tubes (BD Vacutainer®, BD Company). Group D: Stored at 2–8°C for 7 days in inert separation gel tubes (BD Vacutainer®, BD Company). Group E: Stored at 2–8°C for 7 days in polypropylene aliquot tubes.

The primary instruments used in this trial included an automated chemiluminescence immunoassay analyzer (C3000; Hotgen), a laboratory centrifuge (ThermoFisher Scientific), a 2-8^{o}C refrigerated storage unit (MPC-5V1006; Zhongkeduling), and a -20^{o}C ultra-low freezer (MDF-25V2268E; Zhongkeduling). The assay reagents comprised biological CK18-M30 detection reagents, calibrators, and quality control materials (all from Hotgen). Furthermore, sample collection and processing were performed using VACUETTE 3.5 mL serum tubes containing inert separation gel and 1.5 mL disposable polypropylene aliquot tubes (Kangjian).

All instruments were calibrated according to manufacturer protocols, and reagents were stored at recommended temperatures. CK18-M30 concentrations were measured using the Hotgen C3000 automated chemiluminescence immunoassay analyzer (Hotgen Biotech Co., Ltd., Beijing, China). The assay is based on a sandwich chemiluminescent immunoassay principle using monoclonal antibodies specific for caspase-cleaved CK18 (M30 epitope). The assay was calibrated according to the manufacturer's instructions prior to analysis. Internal quality control materials at two concentration levels were analyzed in each run. The analytical measuring range, limit of detection, and calibration frequency followed the manufacturer's specifications.

At 3 or 7 days, the samples were collected and incubated at RT for 30 min before immediate assay. Results were recorded and compared against baseline for stability per condition. All assays were run on the Hotgen C3000 analyzers, and each run included QC samples. In the laboratory's internal quality control system, the CV is 3%, which is much lower than the manufacturer's inter-batch CV threshold of less than 15%. Experimental samples were submitted for analysis only when the IQC results met the required criteria, i.e., a CV $\leq$ 3%. This strict process ensured the results could be trusted and reproduced—laboratory environment followed by the manufacturer's guideline of the Hotgen C3000 analyzer. The analyzer ran without any error alarms during the test, as confirmed by real-time system log monitoring. All assays used reagents from a single lot (CK18-M30 detection reagent, Lot No. C24060111) to reduce lot-to-lot variation. A standardized approach was used to tightly control pre-analytical and analytical variables, thereby increasing the reliability of comparative stability between storage groups.

The data were analyzed with SPSS Statistics 29. The Shapiro-Wilk test showed that cytokeratin 18-M30 concentrations were not normally distributed in all groups, so the results were presented as median (IQR). Friedman's test was performed across all groups to determine whether there were overall differences, and pairwise Wilcoxon Signed-Rank tests were conducted for each group compared to the control. Statistical significance was set at $P < 0.05$.

Relative deviation threshold: Per reagent manufacturer's instructions, post-storage values deemed stable if ≤±15% deviation from baseline.

Concordance rate: CNAS - CL02 - A003: 2018 for CL, each storage group should have achieved a concordance rate of at least 80%. (China National Accreditation Service for Conformity Assessment., 2021).

Statistical analysis

Groups were considered stable only if they fulfilled both predefined criteria: (1) ≥80% concordance with baseline values and (2) no statistically significant difference compared with baseline ($P \geq 0.05$). Because multiple pairwise comparisons were performed, a Bonferroni-adjusted significance threshold was applied to reduce the risk of type I error. The adjusted P-value threshold was calculated based on the number of pairwise comparisons performed. The study's stability acceptance criteria and concordance thresholds align with the bioanalytical validation principles commonly used in clinical medication

trials and pharmaceutical research. The ±15% deviation threshold is consistent with commonly accepted bioanalytical method validation criteria for ligand-binding assays, where deviations within ±15% are generally considered analytically acceptable. This criterion is considerably lower than the allowable total error requirement for protein analytes in the inter-laboratory proficiency testing organized by the National Health Commission of the People's Republic of China, while also conforming to the detection deviation range specified in the test reagent instructions.(National Health Commission of the People's Republic of China., 2024). Similarly, the ≥80% concordance criterion reflects laboratory accreditation guidance for method comparison and stability verification, indicating that the majority of results remain within predefined acceptable analytical variation. These thresholds were selected to ensure that observed variations would not meaningfully alter clinical interpretation of CK18-M30 concentrations in routine laboratory or research settings.

RESULTS

The Friedman test indicated a significant overall difference among all storage groups ($P < 0.001$). To further identify specific differences, Wilcoxon signed-rank tests were conducted to compare each experimental group with the baseline group. The results of these tests are as follows:

Group A showed no significant difference from baseline ($P > 0.05$). Concordance reached 100%, meeting validation criteria. Group C showed no significant difference from baseline ($P > 0.05$). With a 90.9% concordance rate, it passed stability assessment. Group B showed a significant difference from baseline ($P < 0.05$), with a 77.3% concordance rate below the ≥80% threshold. Group D had a significant difference from baseline ($P < 0.05$), with a 72.7% concordance rate below the required standard. Group E had a significant difference from the baseline group ($P < 0.01$), with a concordance rate of only 40.9%. The results are shown in table 1.

Stability after 3-day storage under different conditions

Comparison of Group A with baseline, 12 sample results below baseline with relative deviations of 2.1%–14.7%. 10 sample results above baseline with relative deviations of 2.2%–14.3%. 6 sample results >10% deviation (all with baseline CK18-M30 <150 U/L).

Comparison of Group B with baseline, 14 sample results below baseline with relative deviations of 1.2%–24.5%. 8 sample results above baseline with deviations of 0.1%–8.3%. All 7 samples with >10% deviation were below baseline, with baseline cytokeratin 18-M30 values of 26.6–1,124 U/. No samples above baseline exceeded 10% deviation.

Comparison of Group C with baseline, 13 sample results below baseline with relative deviations of 0.1%–22.1%. 9 sample results above baseline with deviations of 0.5%–13.7%. 6 Samples with >10% deviation, only Sample number 10 had a baseline value >500 U/L and the remaining five Samples were <150 U/L. The results are shown in table 2.

Stability after 7-day storage under different conditions

Comparison of Group D with baseline,16 sample results below baseline with relative deviations of 0.4%–31.7%, including 6 exceeding the ±15% threshold (all with baseline CK18-M30 <150 U/L).6 sample results above baseline with relative deviations of 0.2%–12.9%, all within the ±15% acceptable range.

Comparison of Group E with baseline, 18 sample results below baseline with relative deviations of 2.5%–36.9%, including 12 exceeding 15% (baseline CK18-M30: 26.6–1,124 U/L).4 sample results above baseline with relative deviations of 2.8%–32.3%, with one sample exceeding 15%. The results are presented in Table 3.

A comparison of the five storage groups with the baseline values reveals that the absolute deviations for Groups A, B, and C were all close to zero, consistent with their concordance rates. Group A demonstrated the least variability from baseline, while Group E exhibited the greatest absolute deviation and variability (Fig. 1).

With relative deviation, Group C demonstrated the closest proximity to zero, while Group A exhibited the least variability in relative deviation. Conversely, Group E exhibited the most substantial relative deviation and variability (Fig. 2).

DISCUSSION

Human cytokeratin 18 fragments have become increasingly clinically applied over recent years, especially in the assessment of fibrosis severity among people with NASH and NAFLD (Kamada *et al*., 2026). And also in showing its great clinical value for the diagnosis and prognosis in AH, ALF and chronic viral hepatitis (Gomez-Rioja *et al.*, 2023; Im, 2023). As well as being included in the guidelines and treatments from different countries (Kulkarni *et al.*, 2026). The current literature has shown that most laboratory errors occur during the preanalytical process (Wei *et al*., 2023; Winter *et al*., 2023). Therefore, it is very important to ensure the quality of the preanalytical stage. Based on previous studies, we selected clinical samples covering low, medium and high concentrations, with more than 20 samples in each category, to reach the medical decision level (Gomez-Rioja *et al*., 2023). A study about the influences of distinct storage circumstances on the stability of CK18-M30 in serum, providing data - backed outcomes that are valuable.

Table 1: Comparison of baseline and experimental groups.

Group	Number	Results (U/L) M (P25,P75)	Z value	P value	Concordance rate (%)
Baseline	22	55.8 (111.4,237.8)			
A	22	52.9 (117.8,240.7)	-0.52	0.60	100%
B	22	52.6 (107.3,236.2)	-2.00	0.046	77.3%
C	22	53.7 (104.9,248.6)	-0.29	0.77	90.9%
D	22	55.4 (105.8,225.7)	-2.09	0.036	72.7%
E	22	50.5 (104.9,205.9)	-2.87	0.004	40.9%

Note: M: Median; P25: 25th percentile; P75: 75th percentile

Table 2: Detailed comparison of relative deviations from baseline.

Sample	Baseline results	A		B		C	
		Results (U/L)	Diff. (U/L)/ AD (%)	Results (U/L)	Diff. (U/L)/ AD (%)	Results (U/L)	Diff. (U/L)/ AD (%)
1	72.9	62.2	-10.7/-14.7	62.8	-10.1/-13.9	65.3	-7.6/-10.4
2	31.9	28.9	-3.0/-9.4	25.0	-6.9/-21.6	29.3	-2.6/-8.2
3	60.4	55.0	-5.4/-8.9	57.8	-2.6/-4.3	58.4	-2.0/-3.3
4	26.6	23.3	-3.3/-12.4	22.1	-4.5/-16.9	23.8	-2.8/-10.5
5	40.5	38.4	-2.1/-5.2	36.8	-3.7/-9.1	39.7	-0.8/-2.0
6	441.2	425.7	-15.5/-3.5	405.2	-36.0/-8.2	440.2	-1.0/-0.2
7	84.8	86.7	1.9/2.2	84.9	0.1/0.1	91.6	6.8/8.0
8	128.3	125.6	-2.7/-2.1	119.4	-8.9/-6.9	118.0	-10.3/-8.0
9	1124.0	1018.0	-106.0/-9.4	995.4	-128.6/-11.4	1076.0	-48.0/-4.3
10	735.6	672.3	-63.3/-8.6	621.0	-114.6/-15.6	609.3	-126.3/-17.2
11	144.5	165.2	20.7/14.3	145.0	0.5/0.3	152.4	7.9/5.5
12	74.5	79.1	4.6/6.2	71.3	-3.2/-4.3	74.4	-0.1/-0.1
13	370.7	359.2	-11.5/-3.1	371.2	0.5/0.1	382.1	11.4/3.1
14	121.3	129.8	8.5/7.0	127.6	6.3/5.2	137.9	16.6/13.7
15	131.7	139.7	8.0/6.1	129.4	-2.3/-1.7	132.3	0.6/0.5
16	90.8	87.0	-3.8/-4.2	94.2	3.4/3.7	92.7	1.9/2.1
17	133.2	147.2	14.0/10.5	104.7	-28.5/-21.4	103.8	-29.4/-22.1
18	1138.0	1114.0	-24.0/-2.1	1203.0	65.0/5.7	1233.0	95.0/8.3
19	101.5	110.1	8.6/8.5	109.9	8.4/8.3	106.0	4.5/4.4
20	193.5	201.3	7.8/4.0	191.2	-2.3/-1.2	204.1	10.6/5.5
21	42.0	46.7	4.7/11.2	31.7	-10.3/-24.5	37.6	-4.4/-10.5
22	27.4	31.2	3.8/13.9	27.8	0.4/1.5	26.8	-0.6/-2.2

However, several limitations should be acknowledged. First, the study included only 22 serum samples collected from individuals undergoing routine physical examination at a single center. Although this number is acceptable for a preliminary stability assessment, the limited sample size may restrict the generalizability of the findings to broader clinical trial or pharmacovigilance settings. Therefore, the results should be interpreted primarily as laboratory-level evidence rather than definitive guidance for large-scale multicenter applications. In addition, lower storage temperatures such as -80°C were not evaluated and should be considered in future studies, particularly for long-term biobanking applications.

Compared to the baseline, according to the experimental data, the samples stored in aliquot tubes at -20°C for 3 days and those stored in separation gel tubes at 2-8°C for 3 days did not show a statistically significant difference.

However, the detected values tended to decline under most storage conditions. This observation may suggest potential analyte degradation or loss during storage; however, no mechanistic experiments were performed in the present study. Possible explanations include proteolytic activity, adsorption to tube surfaces, or other pre-analytical factors, which require further investigation. 3 days -20°C aliquot tubes: all samples had 100% concordance; low-

Table 3: Stability analysis of Groups D and E compared to baseline.

Sample	Baseline results	D		E	
		Results (U/L)	Diff. (U/L) / AD (%)	Results (U/L)	Diff. (U/L) / AD (%)
1	72.9	67.5	-5.4/-7.4	63.1	-9.8/-13.4
2	31.9	24.6	-7.3/-22.9	22.6	-9.3/-29.2
3	60.4	60.5	0.1/0.2	54.1	-6.3/-10.4
4	26.6	23.3	-3.3/-12.4	22.1	-4.5/-16.9
5	40.5	40.2	-0.3/-0.7	39.5	-1.0/-2.5
6	441.2	382.4	-58.8/-13.3	354.7	-86.5/-19.6
7	84.8	92.2	7.4/8.7	88.2	3.4/4.0
8	128.3	130.4	2.1/1.6	116.2	-12.1/-9.4
9	1124.0	973.5	-150.5/-13.4	787.1	-336.9/-30.0
10	735.6	664.1	-71.5/-9.7	561.7	-173.9/-23.6
11	144.5	111.6	-32.9/-22.8	110.9	-33.6/-23.3
12	74.5	68.6	-5.9/-7.9	61.2	-13.3/-17.9
13	370.7	363.6	-7.1/-1.9	341.7	-29.0/-7.8
14	121.3	120.8	-0.5/-0.4	113.2	-8.1/-6.7
15	131.7	110.2	-21.5/-16.3	105.4	-26.3/-20.0
16	90.8	102.5	11.7/12.9	120.1	29.3/32.3
17	133.2	91.0	-42.2/-31.7	84.0	-49.2/-36.9
18	1138.0	1225.0	87.0/7.6	1176.0	38.0/3.3
19	101.5	109.0	7.5/7.4	104.3	2.8/2.8
20	193.5	179.7	-13.8/-7.1	160.6	-32.9/-17.0
21	42.0	30.2	-11.8/-28.1	28.1	-13.9/-33.1
22	27.4	22.9	-4.5/-16.4	21.8	-5.6/-20.4

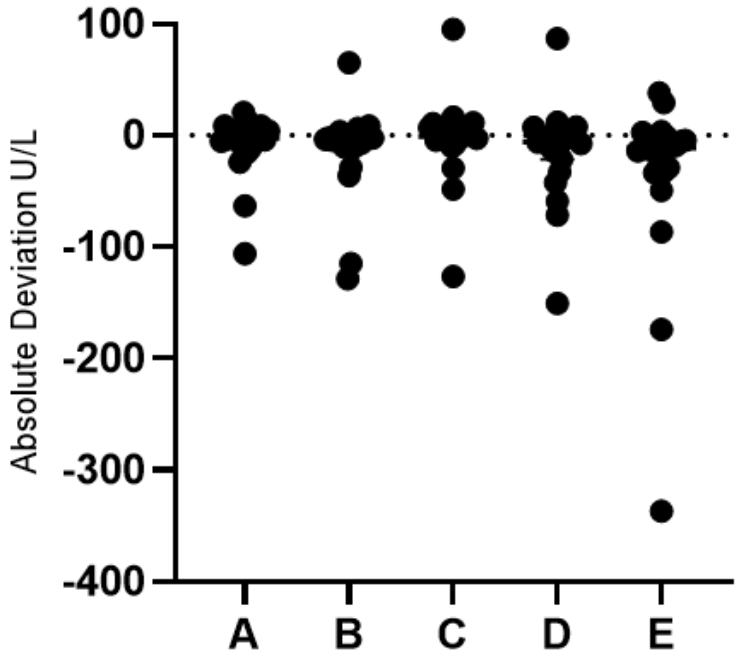

Fig. 1: Absolute deviation (U/L) of serum CK18-M30 concentrations under different storage conditions compared with baseline values. Twenty-two serum samples were stored under five predefined conditions: Group A (−20°C for 3 days, polypropylene aliquot tubes), Group B (2–8°C for 3 days, polypropylene aliquot tubes), Group C (2–8°C for 3 days, separation gel tubes), Group D (2–8°C for 7 days, separation gel tubes), and Group E (2–8°C for 7 days, polypropylene aliquot tubes). Absolute deviation was calculated as: Post-storage concentration (U/L) − Baseline concentration (U/L).

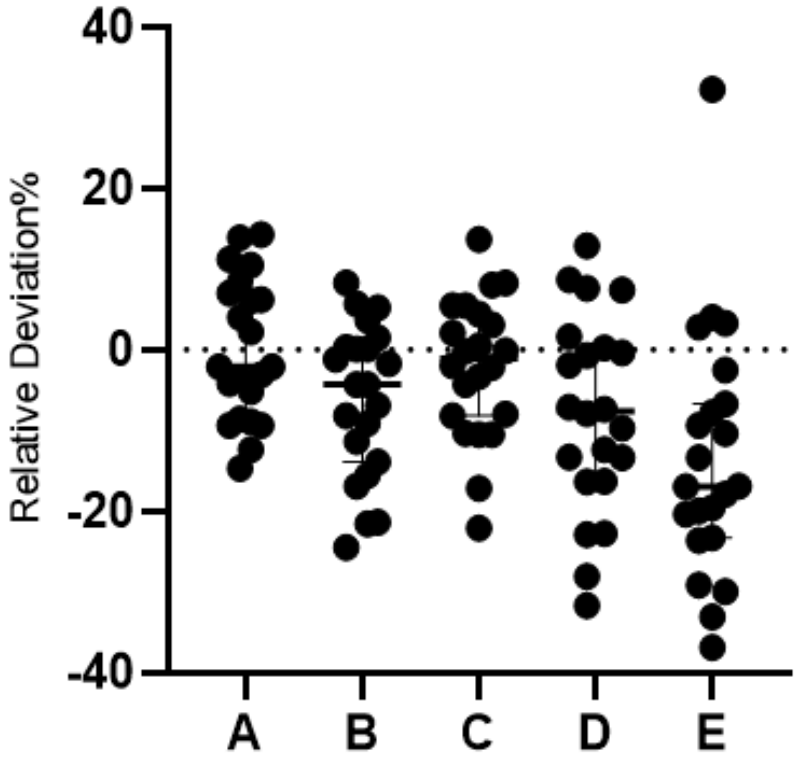

Fig. 2: Relative deviation (%) of serum CK18-M30 concentrations following storage under different temperature and tube conditions compared with baseline values. Relative deviation was calculated as: [(Post-storage concentration − Baseline concentration) / Baseline concentration] × 100%. CK18-M30 concentrations are expressed in U/L. Storage groups and temperature conditions are identical to those described in fig. 1.

temperature storage lowers enzyme activity and reaction rate, as per the reagent instructions' recommendations. At temperatures below 2-8°C, separation gel tubes performed better than aliquot tubes for concordance and stability. The use of separation gel tubes can stabilize the sample to some extent, consistent with previous studies (Verma *et al.*, 2026; Li *et al*., 2023). On the one hand, the contents of the separation gel tube can stabilize CK18-M30; on the other hand, since the aliquot tubes are not specifically labeled as sterile, microbial degradation of CK18-M30 may have occurred. Notably, aliquot tubes stored at 2-8°C for 7 days had values below baseline, with substantial reductions. Experimental data show that the original separation gel tubes are stable at 2-8°C for 3 days or less, thus supporting the storage of untested samples on the same day in the laboratory and can be stored for a longer time while maintaining stability (Meza *et al*., 2025; Xu *et al*., 2026).

Practical implications for laboratory and clinical research settings

From a laboratory perspective, the stability of CK18-M30 is relevant for institutions performing delayed sample analysis or centralized testing (Zhang *et al*., 2024). The present findings provide practical guidance for hospital laboratories and clinical research units regarding acceptable short-term storage conditions. However, given that this study was conducted at a single center using one analyzer platform and a single reagent lot, extrapolation to regulatory policy or large-scale pharmacovigilance frameworks should be made cautiously. Multicenter validation studies using different analytical systems would be necessary before broader implementation.

CONCLUSION

In conclusion, serum CK18-M30 demonstrated acceptable short-term stability when stored at −20°C for 3 days and in separation gel tubes at 2–8°C for up to 3 days. Storage at 2–8°C in polypropylene aliquot tubes beyond 3 days resulted in significant deviations from baseline values. These findings provide laboratory-level evidence to support appropriate pre-analytical handling of CK18-M30 when immediate analysis is not feasible. Further multicenter studies involving larger and clinically diverse populations are warranted to confirm these observations.

Acknowledgments

Not applicable.

Authors' contributions

Zheng GW contributed to conceptualization, data analysis, manuscript drafting and revision. Xia XP supervised the study implementation and provided methodological oversight. Jia FL performed the experimental procedures

and laboratory analyses. Li WX was responsible for sample collection and data management, while Wang J contributed to data collection and statistical processing. Li JY provided technical support and coordinated laboratory operations. All authors reviewed and approved the final version of the manuscript.

Funding
This work was supported by the Department of Clinical Laboratory, the Fourth Affiliated Hospital, Zhejiang University School of Medicine, Yiwu, China.

Data availability statement
All data generated or analysed during this study are included in this published article (and its supplementary information files).

Ethical approval
This study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Ethical Review Board of the Fourth Affiliated Hospital, Zhejiang University School of Medicine (Approval No. 2025218; approved September 1, 2025). This study was performed in adherence with the STROBE guidelines. See supplementary file for the STROBE checklist.

Conflict of interest
The author(s) declare that they have no conflicts of interest related to this work.

Supplementary data

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