Research on the stability changes in expert consensus of the ACTH detection preprocessing scheme

https://doi.org/10.1515/labmed-2023-0086
Received July 16, 2023; accepted December 14, 2023; published online January 5, 2024

Abstract

Objectives: Adrenocorticotropic hormone (ACTH) is extremely unstable and can easily degrade at room temperature. The experts agreed that the samples should be transported in an ice bath. If it cannot be detected immediately, the plasma should be separated and frozen, which is difficult to carry out in routine practice. This study was performed to explore the preanalytical factors that influence the stability of adrenocorticotropic hormone (ACTH) measurements.

Methods: ACTH levels in 21 EDTA whole-blood samples were measured immediately after 0 h and then divided into three equal groups according to the corresponding values (low, L; median, M; and high, H). Next, three sample processing methods (including seven subtypes) were used: whole blood was uncentrifuged (named the A method), stored at 4 °C or 22 °C, centrifuged but not subjected to plasma removal (the B method), stored at 4 °C or 22 °C, and centrifuged with the plasma removed and stored (the C method) at 4 °C, 22 °C, and −20 °C. Each subtype contained three samples, namely, L, M, and H; these samples were retested using a Siemens XP2000 at different times. The change bias was calculated at 0 h.

Results: Compared to that at 0 h, there was no significant change in ACTH up to 24 h when the sample was stored at 4 °C or 22 °C with the B method (p>0.05), while it significantly changed (up or down >10%) at 4 °C/24 h (bias is expressed as the mean±SEM; 13.37±21 %, p<0.05) and 22 °C/12 h (9.13±7.68 %, p<0.05) with the A method; and 4 °C/24 h (8.93±5.54 %, p<0.05), 22 °C/12 h (9.5±4.47 %, p<0.05) and −20 °C/3 h (12.03±4.8 %, p<0.05) with the C method.

Conclusions: After ACTH samples were centrifuged, the presence of plasma without removal did not affect the detection value, and the sample was stored at 4 °C for up to 24 h. There was a significant difference in the detection of ACTH when the sample was stored at −20 °C and thawed again (p<0.05).

Keywords: adrenocorticotropic hormone; centrifugation; temperature; time; stability

Introduction

Clinically, adrenocorticotropic hormone (ACTH) is an important indicator for the diagnosis of Cushing’s disease [1, 2], adrenal insufficiency [3], hypophysis, and other diseases [4] and can also assist in the diagnosis of secondary hypertension [5]. ACTH is characterized by poor stability, low immunogenicity, and easy degradation by proteolytic enzymes, which can worsen its stability; therefore, the expert consensus in China recommends that blood samples be stored on ice/water immediately after collection, centrifuged in a cooled centrifuge, analyzed within 1 h of collection, and stored at −80 °C if it cannot be detected immediately [6]. However, maintaining a constant low temperature from sampling to testing is not easy in routine laboratory practice. In our laboratory, we followed the following procedure: if the sample could not be tested immediately, the sample was centrifuged, the plasma was collected and stored at −20 °C, and the sample was subsequently thawed the next day for testing.

To obtain an accurate ACTH result, it is necessary to study preanalytical interference factors. There are divergent views on the factors that interfere with ACTH detection. At
the same temperature, there has been disagreement about the duration of ACTH stability. Mette Christensen et al. reported that the level of ACTH in whole blood was not affected by exposure to room temperature for 4 h [7]. Furthermore, Vijayalakshmi et al. confirmed that the ACTH concentration was not affected by EDTA-plasma at room temperature for 12 h [8]. The stability of ACTH also differed at different temperatures irrespective of whether the plasma was centrifuged. Wu et al. revealed that whole blood temperature was affected by room temperature for 4 h but not by room temperature for 8 h [9]. Kong et al. reported that in centrifugally separated EDTA-plasma or nonseparated plasma (for centrifugal separation of plasma, EDTA-plasma was stored at 4 °C or 22 °C; for nonseparated plasma, the plasma was not removed following centrifugation and was left sitting on top of the cell layer without any gel layer separating it) at 4 °C or 22 °C, ACTH can be stable for 12 h; however, in whole blood at 4 °C or 22 °C, ACTH stability does not exceed 2 h [10]. Xi et al. reported that although the detection value of ACTH decreased over time, there was no statistically significant difference between plasma collected within 24 h and preserved plasma (preservation methods: EDTA-plasma was placed at 22 °C for 2, 3, 4, 8, or 24 h; at 4 °C for 4, 8, or 24 h; or at −20 °C for 24 h) [11].

Given the lack of clarity from the results of previous studies, as described above, this study was performed to rework and verify the potential influencing factors of ACTH stability and to design the most comprehensive and rigorous group processing method to provide a more accurate basis.

Materials and methods

Samples and design

This study was approved by the Ethics Committee of Tianjin Medical University General Hospital (No. IRB2022-wz-055). A total of 21 EDTA-K$_2$ whole-blood samples (from patients who had visited the clinic for ACTH testing) were collected from the clinical laboratory of our hospital. There was no haemolysis, lipemia, particulate matter, or microbial contamination in the samples. The patient had a normal ACTH level in the reference range <10.21 pmol/L (46 pg/mL, unit of Immulite-2000 XPI Analyser), and the instrument detection limit was 1.11–57.72 pmol/L (5–260 pg/mL). We defined 2.66–4.44 pmol/L (12–20 pg/mL) as the low group (L), 4.44–10.21 pmol/L (20–46 pg/mL) as the median group (M), and 10.21–57.72 pmol/L (46–260 pg/mL) as the high group (H). Samples were selected according to the ACTH value according to the principle of L, M, and H trisection (seven patients in each). Three sample processing methods (plus different storage temperatures for a total of seven subtypes) were used (as shown in Figure 1 and Table 1): whole blood was centrifuged just prior to analysis (named method A), and the plasma was stored at 4 °C or 22 °C; Blood was centrifuged, and the plasma was separated from the cells but was left sitting on the cell layer following centrifugation and not removed (method B), the sample was stored at 4 °C or 22 °C; and the blood was centrifuged and the separated plasma was collected (method C) and stored at 4 °C, 22 °C, or −20 °C. A set of ACTH samples containing one L, M, or H sample each was retested at different delay time points (3, 6, 12, and 24 h).

Measurement

All the ACTH concentrations were measured in triplicate using a Siemens automatic immunochemical analyser (Immulite-2000 XPI, Siemens, Germany) according to the manufacturer’s instructions. Before detection, parameters such as calibration of the reagents, quality control, and precision of the instruments were ensured. During the experiment, the quality control coefficient (CV) of ACTH on this device was approximately 8 %, meeting the quality control requirements. The change bias was calculated according to the testing value at 0 h.

Statistical analysis

The change in ACTH concentration percentage (ΔACTH%) is expressed as the mean ± SEM. A change >10 % compared with the baseline value was considered clinically relevant and was used as the threshold for evaluating stability; values <10 % were unlikely to be clinically significant [7–9]. The change bias was calculated using the following equation: bias=ACTH concentration at time x−the ACTH concentration at time 0 h/the ACTH concentration at time 0 h×100 %. Changes in concentrations

Figure 1: Samples and processing methods.
were analysed with ANOVA or paired t tests using SPSS 22.0 (SPSS, Inc., USA) software, and p<0.05 was considered to indicate statistical significance.

Results

In method A (whole blood, uncentrifuged), when the samples were stored at 4 °C for 24 h, the average bias was approximately 13.37±21 %, with a change in possibility of >10 %, which was mainly attributed to the L (25 %) and M (−10.4 %) groups. When stored at a temperature of 22 °C, the average bias (9.13±7.68 %) at 12 h was >10 % (mainly affected in the M group, value of −17.5 %); at 24 h, the average bias rose to 15.1±8.43 % >10 % and was mainly affected in the L (−19.3 %) and M (−20.6 %) groups. In method B (centrifuged, unremoved plasma), at 4 °C and 22 °C for up to 24 h, there was no mean bias >10 %, regardless of the L, M, or H levels. In method C (centrifuged, separated plasma removed), similar to method A, the average bias was >10 % at 4 °C for 24 h and was mainly affected by the L value (14.8 %). After treatment at 22 °C for 12 h, the average bias also decreased to <10 %, which was mainly affected by the M value (−14.2 %). Under the −20 °C storage condition, the average deviation was >10 % at 3 h (12.03±4.80 %) and increased at 6 h (6±6.49 %), 12 h (5.93±4.70 %), and 24 h (7.13±9.59 %).

The details of the above bias calculation results are shown in Table 1, and the original test values are presented in Supplementary Tables 1–3. Figure 2 shows the variation trend of ACTH values under different temperature conditions and sample processing methods, which was plotted according to the values in Table 1.

Discussion

In this study, the effects of centrifugation, the methods used for the separation and removal of plasma, the storage time, and the storage temperature on the ACTH concentration were investigated. In comparison to previous studies, we were more rigorous in sample enrolment, and ACTH samples with different baseline values of L, M, and H (divided into different levels, similar to the quality control in daily clinical work) were used in the study of different processing schemes [7–9]. Our key results include the following: (1) There was no clinically significant difference within 12 h, irrespective of the sample processing method. (2) In method B, centrifuging blood but not removing plasma was the best processing method for ensuring the stability of the ACTH concentration within 24 h. (3) Freezing-thawing (−20 °C storage) affected the stability of the ACTH concentration.

In method A, the unstable bias of the whole blood could have been caused by the following reasons: the samples were not centrifuged, the plasma and blood cells were mixed, and ACTH was degraded by plasma protease, with a large change rate, which was similar to the findings of the expert consensus on Cushing’s syndrome (ACTH is rapidly degraded by plasma proteases) [12]. In method B, the blood sample was centrifuged, but the plasma was not removed; surprisingly, the change in values at 4 °C or 22 °C and
different storage times was <10 %, indicating increased stability (for a ratio >10 % bias >0/8) compared to that in methods A (3/8) or C (7/12), with p <0.05. However, we were unable to find any relevant references to further evaluate these findings. Guo et al. reported that ACTH levels in blood are correlated with various blood cells and related cytokines, especially with IgA levels [13]. Based on this, it can be speculated that, by centrifugation without removal of plasma, the underlying blood cells may continue to provide relevant cytokines that maintain ACTH homeostasis. In method C, unlike in methods A and B, the blood sample was centrifuged, and the separated plasma was removed and frozen at −20 °C (the samples in methods A and B could not be frozen at −20 °C because they contained blood cells). It was originally thought that freezing the sample would ensure that the detection value of ACTH would be closer to that in immediately collected plasma. Unexpectedly, the use of stored-thawed plasma resulted in increased bias in ACTH detection. This result contradicts the conventional opinion that freezing offers stability, which forced us to abandon the processing method, which our laboratory had always used for ACTH samples; samples should be stored at −80 °C or −20 °C if they cannot be analysed immediately. This phenomenon has rarely been reported by others. Hillebrand J et al. reported that for most endocrine parameters, multiple freeze-thaw cycles had no effect despite alarming notifications in assay manuals, and repeated freeze-thaw cycling resulted in significant and relevant increases in plasma renin activity and a small decrease in ACTH [14]. Ismail AA further clarified that at −20 °C, slow freezing occurs, unlike ‘snap-freezing’, which occurs when using dry ice or liquid nitrogen at −70 °C and has a structural impact on macromolecular hormones, possibly affecting the stability of subsequent detection [15].

Compared with those of previous studies [7–11], the influencing factors explored in this study were more comprehensive. Slightly different from most of the previous studies, we used a Siemens rather than a Roche detection system; however, we believe that this approach did not prevent us from exploring the effects of sample processing. However, further research may be needed to confirm these findings in different laboratory settings and with various measurement systems. In addition, we proposed that storage at −20 °C has an effect on ACTH detection. In the conventional ACTH sample processing method, the plasma needs to be removed, stored at −20 °C, and then tested after being thawed the next day. However, in this series of operations, the steps of processing were complicated; plasma centrifugation and transfer operations could increase the risk of aerosol infection, and the presence of too many samples increased the possibility of confusion or mislabelling when transferring samples into other/new tubes.

Figure 2: ACTH stability under different conditions. (A) ACTH stability within 24 h according to method A. (B) ACTH stability within 24 h according to method B. (C) ACTH stability within 24 h according to method C. The dotted lines in (A, B and C) represent the warning line for 10 % bias.
In conclusion, our data indicate that centrifugation without removal of plasma followed by storage at 4 °C has no effect on changes in ACTH levels, even after 24 h; thus, this process is the best processing method and is worth promoting. Moreover, ACTH was detected at −20 °C, after which the sample was thawed again. The current processing guidelines recommended for ACTH immunoassays seem dispensable, and less stringent parameters should be acceptable. Our findings could reduce the burden on clinical staff and facilitate easy detection of ACTH in hospital settings.

**Research ethics:** Conform “International ethical guidelines for biomedical research involving human subjects (2002)” developed by Council For International Organizations Of Medical Sciences (CIOMS) in collaboration with World Health Organization (WHO), Declaration of Helsinki (as revised in 2013) researches in this article are approved. Ethical No. IRB2022-WZ-055.

**Informed consent:** Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

**Author contributions:** Wei Luo and Chunhui Yuan designed the study. Wei Luo, Tianqi Hao, Yamin Chai, Lizhen Dong and Jiawei Chen carried out the experiments, Wenbin Tuo and Zichao Jia performed the analysis. All authors read and approved the final version of the manuscript.

**Competing interests:** All authors state no conflict of interest.

**Research funding:** Youth Incubation Fund of Tianjin Medical University General Hospital (No. ZYYFY2016024), New Century Talent Program of Tianjin Medical University General Hospital (No. 209060102501), Tianjin Health Technology Project (No. TJWJ2023MS003) and the Natural Science Foundation of Wuhan Science and Technology Bureau (2022020801020573).

**Data availability:** The raw data can be obtained on request from the corresponding author.

**References**


**Supplementary Material:** This article contains supplementary material (https://doi.org/10.1515/labmed-2023-0086).