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Research Article

Comparability of adrenocorticotropic hormone measured in spray-dried K2-EDTA and spray-dried K3-EDTA tubes

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Abstract

Objectives: Blood samples for adrenocorticotropic hormone (ACTH) tests are collected in dipotassium or tripotassium ethylenediaminetetraacetic acid (K2-EDTA or K3-EDTA) tubes. The interchangeable use of spray-dried K2-EDTA and K3-EDTA tubes may occasionally occur in practice and is appropriate only if both tube types yield comparable analytical results. Therefore, this study aimed to evaluate the interchangeability of these tubes for ACTH measurement.

Methods: Seventy-two volunteers were included in the study. From each participant, K2-EDTA and K3-EDTA blood samples were collected simultaneously. ACTH was measured by chemiluminescent immunoassay on a Maglumi X3 analyzer (SNIBE, Shenzhen, China). The Wilcoxon signed-rank test, Passing-Bablok regression, Mountain plot, and Bland-Altman analyses were used to evaluate differences between measurements.

Results: The median ACTH concentration in K2-EDTA tubes was 35.1 pg/mL (10.35–107.16) [7.73 pmol/L (2.28–23.60)] and in K3-EDTA tubes was 32.96 pg/mL (9.85–99.90) [7.26 pmol/L (2.17–22.00)]. The Wilcoxon signed-rank test ($z=-7.14$, $p<0.001$) demonstrated a statistically significant difference between the results. In the Bland-Altman analysis, the mean difference between the two samples was determined to be 1.49 pg/mL (0.33 pmol/L). Lin's concordance correlation coefficient was 0.989 (95% CI: 0.984–0.993), indicating good agreement between ACTH results obtained from the two tubes. Mountain plot analysis demonstrated that most differences clustered around zero, with an asymmetric right tail suggesting the presence of larger differences in a limited number of samples.

Conclusion: ACTH concentrations measured in both tubes are proportional, and the results obtained from K2-EDTA and K3-EDTA tubes support analytical comparability within the ACTH concentration range evaluated in this study. However, complete interchangeability between the results of the two tube types cannot be assumed in all clinical scenarios; further studies are needed in subjects with higher ACTH levels.

Keywords: Adrenocorticotropic hormone, bias, K2-EDTA; K3-EDTA, pre-analytical phase, test tube comparison

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Human adrenocorticotropic hormone (ACTH) is the stimulator of glucocorticoid synthesis and secretion by the adrenal cortex. Accurate measurement of the plasma ACTH concentration is crucial for diagnosing disorders of the hypothalamic–pituitary–adrenal axis, including Cushing's syndrome and adrenal insufficiency [1, 2]. Meanwhile, pre-analytical factors such as sample type and storage time have long been recognized as major determinants of ACTH assay reliability [3–7]. Among the pre-analytical variables, the blood collection process, including tube selection, plays a critical role [6, 8]. The type and concentration of additives in blood collection tubes can significantly influence test results by affecting sample stability, clotting, or analyte concentration. Therefore, proper selection and validation of blood collection tubes are essential steps in minimizing pre-analytical variability and ensuring accurate and reliable laboratory outcomes [8, 9].

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In our laboratory, ACTH concentrations are measured using a chemiluminescent immunoassay method on a Maglumi X3 analyzer (SNIBE, Shenzhen, China). Ethylenediaminetetraacetic acid (EDTA) tubes are reported by the manufacturer as the preferred anticoagulant according to the ACTH analysis sheet. EDTA is present in blood collection tubes as the dipotassium (K2), tripotassium (K3), or sodium EDTA salts; however, no major international guideline specifically recommends K2-EDTA over K3-EDTA, and both tube types are considered acceptable for ACTH measurement [10].

Our laboratory is a large, multi-site facility formed by the merger of laboratories operating at different locations. The interchangeable use of K2-EDTA and K3-EDTA tubes may occasionally occur in routine practice and is appropriate only if both tube types yield comparable analytical results.

Therefore, this study aimed to evaluate the interchangeability of these tubes for ACTH measurement.

Materials and Methods

This study was conducted at a University Hospital. The study protocol was approved by the Bursa Yüksek Ihtisas Training and Research Hospital Ethics Committee (Date: 11.03.2026, No: 2024-TBEK 2026/03-03) and carried out in accordance with the Declaration of Helsinki [11]. Written informed consent was obtained from all participants.

The study included healthcare workers and patients presenting to the endocrinology outpatient clinic. In accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) EP09 guideline, 72 participants (36 females, 36 males) aged 18–65 years were included in the study, and no formal sample size calculation was performed (Table 1) [12].

Participants with acute illness, pregnancy, or recent steroid use were excluded.

Venous blood samples were collected from participants between 9:00 a.m. and 10:00 a.m. in the biochemistry laboratory to avoid potential pre-analytical variations related to sample transportation.

Manufacturer instructions for the ACTH assay generally allow the use of both K2-EDTA and K3-EDTA tubes. From each participant, blood was collected consecutively into K2-EDTA (Lot: 3342.0012.24) and K3-EDTA (Lot: 3104.0010.24) tubes (Vacusera; Disera, İzmir, Türkiye), with no predetermined order of tube collection.

The inner wall of the tubes is coated with spray-dried K2 or K3 EDTA rather than a liquid form, which minimizes dilution and maintains the original blood composition, ensuring test accuracy at room temperature (between 22–24°C in our laboratory during the study period). The tubes were filled with approximately 2 mL of venous blood (as recommended by the manufacturer), thus ensuring adequate tube filling and an appropriate blood-to-anticoagulant ratio (1.8 mg per 1 mL of blood).

After specimen collection, we mixed tube contents by inverting them 10 times and centrifuged them at 1500 × g for 10 min (2–8°C) immediately after blood collection. ACTH levels were analyzed within 30 minutes. No hemolysis or lipemia was observed in any of the samples, as assessed objectively using the automated hemolysis and lipaemia indices.

For paired ACTH test results, a maximum allowable bias of ±9.08 pg/mL (±2.0 pmol/L) for concentrations ≤90.82 pg/mL (≤20 pmol/L), or ±10% for concentrations >90.82 pg/mL (>20 pmol/L), was selected based on the Royal College of Pathologists of Australasia (RCPA) allowable limits of performance

Table 1. General characteristics of the study participants and summary of method comparison statistics for ACTH measurements in K2-EDTA and K3-EDTA tubes

Variable	Result
Number of participants	72
Female/Male, n	36/36
Age mean±SD, (range)	29.8±9.4 (18–65)
ACTH concentration, pg/mL (pmol/L), median (range)	
K_2-EDTA tube	35.1 (10.35-107.16) [7.73 (2.28–23.60)]
K_3-EDTA tube	32.96 (9.85-99.90) [7.26 (2.17–22.00)]
Wilcoxon signed-rank test	z=-7.14; p<0.001
Paired samples with higher ACTH in K_2-EDTA, n (%)	69 (95.8)
Passing–Bablok regression analysis	
Regression equation	y=0.086(-0.06 to 0.18) + 1.01×(0.99 to 1.03)
Pearson correlation coefficient	r=0.97; p<0.001
Cusum test for linearity	p=0.68
Agreement analysis	
Mean bias (Bland–Altman)	1.49 pg/mL (0.33 pmol/L)
Spearman correlation analysis	ρ=0.166; p=0.164
Lin's concordance correlation coefficient	0.989 (95% CI: 0.984–0.993)

ACTH: Adrenocorticotropic Hormone; K_2-EDTA: Dipotassium ethylene diamine tetra-acetic acid; K_3-EDTA: Tripotassium ethylene diamine tetra-acetic acid.

for ACTH [13]. This hybrid allowable bias criterion enables a clinically appropriate assessment of analytical performance for ACTH across the measurement range to minimize laboratory-related diagnostic errors.

Plasma ACTH concentrations were analyzed using a commercially available sandwich chemiluminescence immunoassay (Maglumi ACTH kit, SNIBE, Shenzhen, China) on a Maglumi X3 (SNIBE, Shenzhen, China) analyzer. Manufacturer-provided reagents, calibrators, and internal quality control materials were used. Quality control materials at two concentrations [35 and 200 pg/mL (7.71 and 44.04 pmol/L)] were analyzed daily in duplicate for 20 days. Our laboratory participates in the Serocon External Quality Control Program (Konya, Türkiye) during the study interval.

Statistical Analysis

MedCalc statistical software (MedCalc, Mariakerke, Belgium) was used to analyze the data. Data normality was tested using the Kolmogorov–Smirnov test. Agreement and differences between ACTH concentrations measured in K2-EDTA and K3-EDTA tubes were analyzed using the Wilcoxon signed-rank test, Passing–Bablok regression, Spearman correlation analysis, Mountain plot (folded empirical cumulative distribution plot), and Bland–Altman analysis. The strength of agreement between the two results was also assessed using Lin's concordance correlation coefficient (CCC), which incorporates both precision and accuracy [14]. Statistical significance was defined as $p<0.05$.

Results

In day-to-day quality control assessments, the coefficient of variation (CV) for ACTH measurements was 3.99% for QC1 [(35 pg/mL; range 24.5–45.5 (7.71 pmol/L; range 5.4–10.01)] and 2.67% for QC2 [200 pg/mL; range 140–260 (44.04 pmol/L; range 30.83–57.26)].

The median ACTH concentration from 72 participants in K2-EDTA tubes was 35.1 pg/mL (10.35–107.16) [7.73 pmol/L (2.28–23.60)] and in K3-EDTA tubes was 32.96 pg/mL (9.85–99.90) [7.26 pmol/L (2.17–22.00)]. The Wilcoxon signed-rank test (z=-7.14, $p<0.001$) demonstrated a statistically significant difference between the two tubes, showing a systematic positive bias for ACTH results from K2-EDTA tubes. In this study, the ACTH result obtained from 69 K2-EDTA tubes was higher than the result obtained from K3-EDTA tubes.

The regression line equation [y=0.086+1.01x; (95% CI for intercept -0.06 to 0.18 and for slope 0.99 to 1.03)] and Pearson correlation coefficient (r=0.97, $p<0.001$) indicated a strong relationship between ACTH results from the two tubes (Fig. 1). The Cusum test for linearity (p=0.68) showed no significant deviation from linearity (Table 1). The 95% CI for the intercept includes zero, indicating that there is a small constant difference between the results obtained from the two specimen collection tube types. The 95% CI for the slope includes a value of one, showing no proportional difference between paired ACTH results and indicating that the measurement bias did not increase systematically with increasing ACTH concentrations [15].

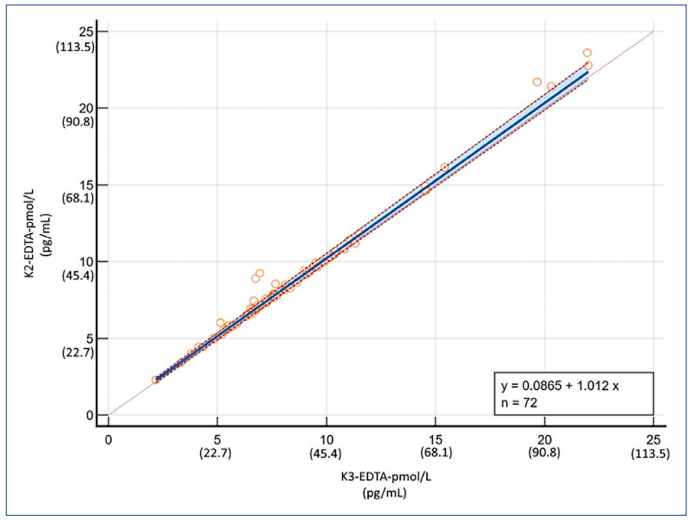

Figure 1. Passing-Bablok regression analysis of ACTH concentrations measured.

Passing-Bablok regression analysis of the comparability of ACTH concentrations measured in K2-ethylenediaminetetraacetic acid (EDTA) and K3-EDTA Vacusera tubes (Disera, İzmir, Türkiye). Solid line represents regression line; dashed lines represent confidence interval (CI) for the regression line; dotted line is the line of identity (x=y). n=72; concentration range 9.85-107.16 pg/mL (2.17-23.60 pmol/L); Pearson correlation coefficient r=0.97, $p<0.001$. Regression line equation: y=0.086+1.01x; 95% CI for intercept -0.06 to 0.18 and for slope 0.99 to 1.03 indicated good agreement.

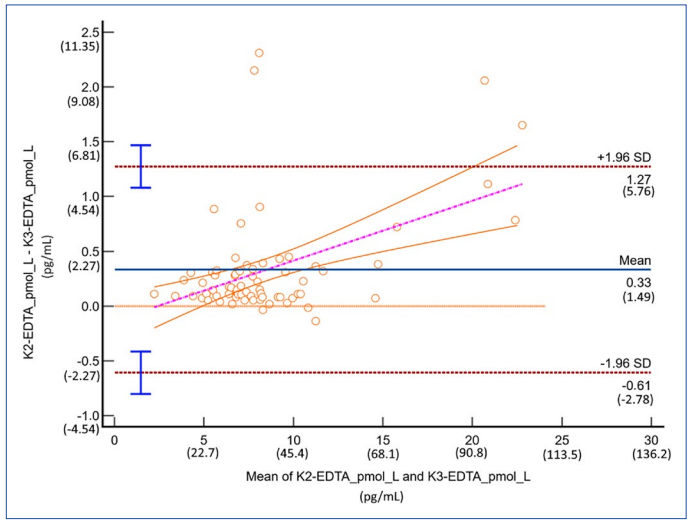

Figure 2. Bland-Altman plot of the comparability of ACTH measurements.

Solid lines represent mean difference and the outer dotted lines represent limits of agreement (mean difference ±1.96 SD). K2-EDTA: Dipotassium ethylene diamine tetra-acetic acid tubes K3-EDTA, tripotassium ethylene diamine tetra-acetic acid tubes. ACTH: Human adrenocorticotropic hormone.

Bland–Altman analysis demonstrated a mean difference of 1.49 pg/mL (0.33 pmol/L) between the ACTH results obtained from K2-EDTA and K3-EDTA tubes (Table 1). This bias is lower than the maximum allowable bias of ±9.08 pg/mL (2.0 pmol/L), indicating acceptable agreement for ACTH concentrations between 9.85 and 107.16 pg/mL (2.17 and 23.60 pmol/L) (Fig. 2). However, the analysis showed that the two measurements do

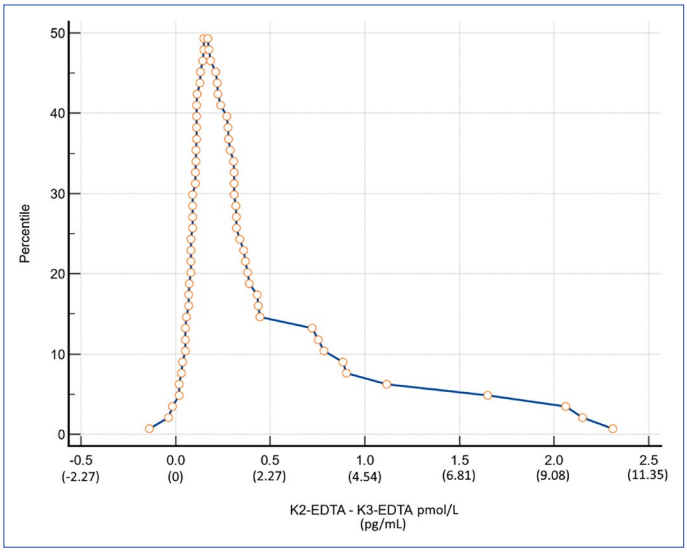

Figure 3. A mountain plot (or "folded empirical cumulative distribution plot") of the ACTH measurements.

Percentile for each ranked difference between ACTH measurements of K2-EDTA tubes and K3-EDTA tubes. K2-EDTA: Dipotassium ethylene diamine tetra-acetic acid tubes K3-EDTA, tripotassium ethylene diamine tetra-acetic acid tubes. ACTH: Human adrenocorticotropic hormone.

not always agree, and some measurements lay outside the limits of agreement. Spearman correlation analysis showed no statistically significant association between the measurement difference and the mean ACTH concentration (ρ=0.166, p=0.164), indicating that the difference between the two tube types did not increase significantly with increasing ACTH concentrations across the studied range.

Lin's concordance correlation coefficient was 0.989 (95% CI: 0.984–0.993), demonstrating good agreement between the ACTH results obtained from K2-EDTA and K3-EDTA tubes; however, this finding alone does not confirm interchangeability.

Mountain plot analysis demonstrated that most differences clustered around zero, indicating minimal overall bias (Fig. 3). However, the asymmetric right tail suggests the presence of larger differences in a limited number of samples, which may be clinically relevant near decision limits.

Discussion

In the present study, plasma ACTH concentrations in blood samples collected in spray-dried K2-EDTA tubes were slightly higher than those collected in spray-dried K3-EDTA tubes. To the best of our knowledge, no study has yet investigated the comparability of specimens collected in K2-EDTA and K3-EDTA tubes for ACTH measurement. Chang et al. [16] compared the plasma levels of α-synuclein and neurofilament light chain collected in K2- and K3-EDTA tubes and found no clinically significant differences that influenced the measurement of these biomarkers.

Leathem et al. [17] demonstrated that the results of routine blood bank tests performed on 102 blood donor samples and 100 patient samples taken with K2-EDTA and K3-EDTA were equivalent. These results are similar to our finding of close analytical agreement between the two tube types for ACTH measurement within the evaluated concentration range. However, when evaluating disorders of the hypothalamic–pituitary–adrenal axis, ACTH concentrations are often interpreted using clinical decision cut-off points. Consequently, small analytical differences close to these limits can lead to diagnostic variation.

Each molecule of K2-EDTA and K3-EDTA contains two and three dissociable potassium ions, respectively. K3-EDTA contains more potassium per volume, leading to a slightly greater dilution effect, which may explain the lower ACTH levels measured in these tubes [18].

When clinical decisions are based on fixed cut-off values, variability between results becomes especially important.

A maximum allowable bias of ±9.08 pg/mL (for values ≤90.82 pg/mL) [±2.0 pmol/L (for values ≤20 pmol/L)] or ±10% [for values >90.82 pg/mL (>20 pmol/L)] between K2-EDTA and K3-EDTA plasma ACTH measurements was adopted as recommended by the Royal College of Pathologists of Australasia allowable error guidelines [13]. Following these performance limits confirms the acceptable analytical agreement between the results obtained from both tube types, demonstrating that ACTH measurements remain consistent within the studied concentration range. In our study, only 4 volunteers had results greater than 90.82 pg/mL (20 pmol/L). Moreover, in all volunteers, the observed differences in results between these tubes were lower than this predefined criterion.

Bland–Altman analysis demonstrated that the mean difference between the results from the K2-EDTA and K3-EDTA tubes was 1.49 pg/mL (0.33 pmol/L), and the bias exhibited by either method did not exceed the acceptable level of inaccuracy [13].

The combined use of mountain plot visualization and Lin's concordance correlation coefficient provides a robust evaluation of the agreement between the two ACTH measurements. Although the paired results demonstrate good overall agreement, complete interchangeability across all clinical scenarios cannot be assumed [14].

Toprak et al. [19] found that increasing EDTA concentration in blood collection tubes causes a decrease in ACTH concentration. Although their study does not clearly distinguish between K2- and K3-EDTA tubes, the effect of EDTA salt amount is emphasized. They stated that under-filled EDTA tubes should be considered unsuitable for ACTH measurement.

Fraissinet et al. [20] compared ACTH stability in blood collected in K2-EDTA and K3-EDTA+aprotinin tubes. The ACTH concentration in K3-EDTA tubes was higher, which could be explained by the effect of aprotinin rather than the tube type itself. They concluded that pre-analytical factors have a greater impact on ACTH stability than the collection tube type.

Meanwhile, the difference in anticoagulant composition may influence peptide stability, highlighting the importance of standardizing tube selection across laboratories.

Limitations

This study has several limitations. The number of participants was relatively small (n=72), and ACTH concentrations in most volunteers were within the reference interval, with only four participants having ACTH concentrations >90.82 pg/mL (>20 pmol/L). Therefore, our findings may not be generalizable to samples with markedly elevated ACTH concentrations, adrenal insufficiency, Cushing's syndrome, ectopic ACTH syndrome, or samples with values near clinically important decision limits.

Conclusion

In conclusion, ACTH concentrations measured in both tubes are proportional, and the results obtained from K2-EDTA and K3-EDTA tubes support analytical comparability within the ACTH concentration range evaluated in this study.

The limited representation of the higher concentration range also restricts conclusions regarding assay agreement at clinically important decision points, where even small analytical differences between specimen types may have disproportionate clinical consequences, including in the evaluation of ACTH-dependent disorders. Further studies including larger cohorts with higher ACTH concentrations are warranted to confirm these findings. These studies should also address the importance of implementing standardized pre-analytical protocols to ensure reliable analytical results.

Disclosures

Ethics Committee Approval: The study was approved by the Bursa Yüksek Ihtisas Training and Research Hospital Clinical Research Ethics Committee (no: 2024-TBEK 2026/03-03, date: 11/03/2026).

Informed Consent: Written informed consent was obtained from all participants.

Conflict of Interest Statement: None declared.

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