Research Article

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Evaluation of hemolysis interference and possible protective effect of N-phenyl maleimide on the measurement of small peptides

Abstract

Objectives: This study aims to demonstrate that proteolytic enzymes released as a result of hemolysis decrease the small peptide hormones, whereas N-phenyl maleimide (NPM) can be used to prevent hemolysis interference.

Methods: The serum/plasma pools were prepared at two different concentrations and divided into two equal volumes. NPM solution was added to one sample. The erythrocyte package was washed five times and then centrifuged after each wash. Hemolysis was induced by the osmotic shock method upon adding distilled water. 100 µL of hemolysate stock solutions were added over 400 µL of serum/plasma pool. Repeated five times.

Results: Negative interference was demonstrated on ACTH at 200 hemolysis index (HI) for low concentration, at 100 HI and 200 HI for high concentration, and insulin at 100 HI and 200 HI for high concentration. Addition of NPM which inactivates protease enzymes prevented the reduction of ACTH and insulin. No interference was observed at C-peptide and IGF-1 concentration until 200 HI.

Conclusions: Hemolysis was detected to degrade ACTH and insulin, which demonstrated how important the effect of hemolysis can be utilized in deciding whether to accept or reject samples. The addition of NPM can increase accuracy in clinical decisions as well as treatment.

Keywords: ACTH; C-peptide; hemolysis; IGF-1; insulin; interference; maleimide.

Introduction

Hemolysis, which results from the rupture of erythrocytes, causes either negative or positive interference on parameters with different mechanisms. One of these mechanisms is that proteases released from erythrocytes degrade peptide-structured hormones (adrenocorticotrophic hormone (ACTH), insulin, glucagon, calcitonin, parathyroid hormone, and gastrin) and results falsely decreased levels [1]. Previous studies have demonstrated that using protease inhibitors such as, N-phenyl maleimide (NPM) and p-chloromercuriphenylsulfonic acid (pcMPS) to inhibit proteases released from red blood cells reduced the loss of peptide-structured hormones [2, 3].

ACTH contains 39 amino acids, mainly processing the proteolytic cleavage of proopiomelanocortin (POMC), a precursor protein [4]. Since ACTH is sensitive to proteases’ degradation, preanalytical factors such as hemolysis, time, and temperature before plasma separation and analysis affect ACTH results [5]. Preanalytical errors occurring in ACTH measurement, which is of great clinical importance in both Cushing’s syndrome and adrenal insufficiency, can lead to incorrect clinical decisions.

The proinsulin formed by A, B and C chains are synthesized by β cells of Langerhans islets in the pancreas. Proinsulin is divided into 51 amino acid insulin and C-peptide, with an equal mole in proinsulin secretion granules [6]. Measurement of insulin levels is often preferred in investigating the etiology of fasting hypoglycemia, classification of Diabetes Mellitus (DM) subtypes, and insulin resistance among patients [7]. Half of the insulin is extracted in the liver but almost none of the C-peptide. The half-life of C-peptide is longer than that of insulin and its concentration in the blood is higher. Measurement of C-peptide is preferred for evaluating endogenous insulin secretion. It is also used together with insulin and glucose in the differential diagnosis of hypoglycemia (factitious hypoglycemia and hypoglycemia caused by hyperinsulinism). High C-peptide levels can be caused by insulinoma, renal failure, and obesity [8].

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Insulin-like growth factor (IGF)-1 is structurally similar to proinsulin, the precursor of insulin. IGF-1 is a single chain polypeptide consisting of 70 amino acids synthesized in the liver [9].

This study aimed to show the decrease in the small peptides due to proteolytic enzymes released with hemolysis and show how this decrease can be prevented by adding NPM thus, inhibiting protease enzyme activity.

Materials and methods

Collection of samples

Venous blood samples were collected into serum separation tubes (Becton Dickinson and Company [BD], Franklin Lakes, NJ, USA) for insulin, C peptide and IGF-1 tests and ethylenediaminetetraacetic acid (K2EDTA) tubes (Becton Dickinson and Company [BD], Franklin Lakes, NJ, USA) for ACTH. After studying the ordered parameters of patient sample coming to the laboratory, a pool was created from the remaining serum for insulin, C-peptide, IGF-1 and plasma for ACTH. Each pool was divided into two equal volumes, however one of them was treated with the protease inhibitor NPM solution. Necessary care was taken to ensure that the concentrations of selected samples were close to the clinical decision points. Hemolyzed patient samples were not included in the study.

Preparation of hemolysates

Whole blood tube with EDTA was centrifuged at 1,100×g for 10 min and the plasma was replaced with an equal volume of isotonic normal saline (0.9%). Erythrocyte package was washed five times and then centrifuged (1,100×g, 10 min) after each wash. Hemolysis was induced by the osmotic shock method by adding distilled water [10]. After storage at −20 °C for a night, the sample was transferred to room temperature and centrifuged (1,100×g, 10 min). Upon centrifuge, hemoglobin concentration was measured photometrically with Sysmex XN-3000.

Determination of hemolysis index (HI)

As a result of using the hemolysate stock solution, a series of 1000, 500 and 250 mg/dL, hemoglobin concentrations were obtained. Isotonic saline was used for the degree of hemolysis at a concentration of zero. The addition of 100 µL hemolysate stock solution over 400 µL of serum/plasma pools resulted in the creation of HI from 200, 100 and 50 mg/dL. This procedure was repeated five times (n=5).

Preparation of NPM solution

A 400 mM NPM (Sigma-Aldrich, Castle Hill, Australia) stock solution prepared with dimethyl sulfoxide (DMSO) (Merck Millipore, Billerica, MA, USA) was added per milliliter of serum/plasma as 5 µL [2]. No blank effect was detected in the NPM solution.

Analytical methods

ACTH and C-peptide were measured using electrochemiluminescence immunoassay (ECL) on Roche Cobas Elecsys (Roche Diagnostics, Mannheim, Germany). The test principles are based on a one-step sandwich immunoassay. Insulin (DIAsource INS-irma kit KIP1251-KIP1254, Louvain-la-Neuve, Belgium) and IGF-I (IRMA IGF-1 A15729, Immunotech, Marseille, France) concentrations were analyzed using an immunoradiometric assay (IRMA) on gamma counter (PC-RIA MAS; Stratec, Germany).

Statistical analysis

Statistical analysis was performed by SPSS 16.0 package program. Multiple comparisons of HI were performed with repeated-measures ANOVA. When the differences were significant in multiple comparisons, the Bonferroni test was used in post hoc evaluations. On the other hand, Wilcoxon test was used to analyze group differences with and without NPM. Data were presented as median and minimum (min) – maximum (max). Mean values were used when calculating % recovery. Results were considered statistically significant for p<0.05.

Results

The increased degrees of hemolysis in both low and high ACTH concentrations were significantly correlated which proved that we had prepared our samples successfully (p<0.05). A statistically significant decrease in low ACTH concentrations was demonstrated at 200 HI (p=0.011). A compelling increase in ACTH concentrations was shown by adding NPM to 200 HI, where the decline of ACTH due to hemolysis interference was confirmed (Table 1).

Table 1: High and low concentration of ACTH (pg/mL) median values (min–max).

| HI  | ACTH low level | ACTH high level |
|-----|----------------|-----------------|
|     | Without NPM | With NPM | Without NPM | With NPM |
| 15  | (5.69–6.77)  | (6.44–6.85)   | (50.88–52.45) | (51.91–52.92) |
| 50  | 7.69         | 8.31          | 51.0         | 61.03     |
| 100 | (6.90–7.88)  | (8.01–8.51)   | (50.06–51.41) | (60.06–62.51) |
| 200 | (5.92–7.2)   | (7.8–8.29)    | (39.64–41.1) | (57.69–60.08) |

HI, hemolysis index; IS, isotonic saline; NPM, N-phenyl maleimide. Bold values indicate statistically significant. *p<0.05 with multiple comparison and post hoc Bonferroni, †p<0.05 with the comparison of two groups.
A momentous decrease in ACTH levels was detected when creating 100 HI in plasma within high ACTH concentrations. ACTH levels were significantly increased after the addition of NPM at 100 and 200 HI, where significant ACTH reduction due to hemolysis was observable (Table 1).

The increased degree of hemolysis at two different levels significantly reduced insulin results ($p<0.05$). Since the initial point of hemolysis effect could not be detected at low concentrations of insulin, the NPM could not be evaluated as well (Table 2). A significant decrease in insulin was detected after 100 HI, using the post-hoc Bonferroni test at a high insulin concentration. While a significant decrease in insulin due to hemolysis interference was detected at 100 and 200 HI, with the addition of NPM a significant increase in insulin was shown only at 200 HI (Table 2).

No significant decrease due to hemolysis was detected in low and high C-peptide and IGF-1 concentrations up to 200 HI (Tables 3 and 4).

| HI   | Insulin low level | Insulin high level |
|------|-------------------|--------------------|
|      | Without NPM | With NPM | Without NPM | With NPM |
| IS   | 5.71 (5.14–5.88) | 5.32 (5.14–6.31) | 10.41 (10.19–10.67) | 11.44 (9.81–12.62) |
| 50   | 5.53 (4.55–5.87) | 5.30 (4.42–5.80) | 9.56 (8.93–9.89) | 9.63 (8.74–11.70) |
| 100  | 5.27 (4.44–5.62) | 5.11 (4.89–5.54) | (7.14–9.14) | 9.99 (8.14–10.44) |
| 200  | 4.42 (3.59–4.80) | 4.77 (4.09–5.42) | (6.86–8.12) | 7.03 (8.89–10.63) |

| HI   | C-peptide low level | C-peptide high level |
|------|----------------------|----------------------|
|      | Without NPM | With NPM | Without NPM | With NPM |
| IS   | 0.92 (0.91–0.93) | 0.89 (0.88–0.91) | 3.15 (3.13–3.18) | 3.17 (3.11–3.18) |
| 50   | 0.93 (0.93–0.94) | 0.93 (0.92–0.94) | 3.26 (3.23–3.29) | 3.09 (3.03–3.11) |
| 100  | 0.93 (0.93–0.94) | 0.92 (0.92–0.94) | 3.15 (3.09–3.15) | 3.12 (3.08–3.13) |
| 200  | 0.93 (0.92–0.94) | 0.88 (0.87–0.90) | 3.11 (3.08–3.13) | 3.11 (3.06–3.15) |

| HI   | IGF-1 low level | IGF-1 high level |
|------|-----------------|------------------|
|      | Without NPM | With NPM | Without NPM | With NPM |
| IS   | 197.7 (192.8–210.5) | 204.4 (194.9–210.1) | 395.0 (388.3–425.1) | 401.8 (379.6–415.7) |
| 50   | 199.1 (174.5–206.2) | 203.5 (189.0–206.4) | 408.6 (360.8–417.7) | 414.8 (387.2–425.2) |
| 100  | 202.5 (172.9–212.9) | 192.4 (187.6–211.6) | 402.7 (376.2–415.8) | 389.7 (380.7–419.5) |
| 200  | 204.6 (200.9–215.7) | 202.5 (163.0–216.3) | 411.2 (402.3–413.3) | 411.3 (354.5–429.7) |

**Discussion**

Within our study, a significant decrease was found in the 200 HI for low ACTH concentrations. The addition of NPM caused an increase in ACTH concentrations by eliminating the hemolysis effect (Figure 1). When examining the high ACTH concentrations, a significant decrease was detected starting from 100 HI. Whereas ACTH concentrations increased significantly with the addition of NPM at both 100 HI and 200 HI. At a high ACTH concentration, results decreased by 23 and 50% at 100 HI and 200 HI, respectively (Figure 2). However, it has been reported that the Roche kit insert does not interfere with ACTH results up to 400 mg/dL hemolysis. A pathologic high value may fall within the reference range as a result of hemolysis. Hemolyzed samples with close concentrations to clinical decision points may cause low false results to be measured, resulting in undesired clinical decisions, and administering unwanted treatments.

**Table 3:** High and low concentration of C-peptide (ng/mL) median values (min–max).

**Table 4:** High and low concentration of IGF-1 (ng/mL) median values (min–max).

**Figure 1:** Low concentration of ACTH recovery graphic between without NPM and with NPM groups. ACTH concentration decreased to 88% in the group without NPM at 200 HI, where hemolysis was evident. By adding NPM at the same degree of hemolysis (200 HI), the ACTH concentration increased to 111%.
The low concentrations of insulin showed a 24% reduction from baseline at 200 HI (Figure 3). There was a significant decrease due to hemolysis, but it could not be determined from which HI point the hemolysis effect started. There were 83 and 70% decreases in 100 HI and 200 HI of high insulin levels without NPM, respectively. With the addition of NPM, insulin levels at 200 HI increased from 70 to 93% (Figure 4). As a result of this data, it is possible to say that hemolysis reduces insulin concentrations, and the protective effect of NPM could be observed with 200 HI of high insulin levels.

In literature, very few studies investigated the interference of peptide structured hormones and protease inhibitors in preventing hemolysis effect. Livesey et al. found that ACTH concentrations decreased due to hemolysis even at less degree of hemolysis. By adding NPM, the loss of ACTH was recovered [2]. Unlike their research, we prepared two different concentrations of pools, tested different degree of hemolysis and evaluated C-peptide and IGF-1 parameters.

Regarding the effect of NPM on ACTH degradation due to time, the protective effect of NPM was not shown [11]. However, it was detected that N-ethylmaleimide (NEM) protected ACTH from degradation for at least 72 h at room temperature [12]. Aprotinin, a different protease inhibitor, reduced ACTH loss in hemolyzed samples by a similar mechanism [13].

Garinet et al. reported that insulin concentrations decreased with an increasing hemolysate amount, but there was no similar decrease with hemoglobin alone [10]. The most likely cause of negative bias is the insulin-degrading enzyme (IDE) released from erythrocytes [7]. It was stated that for the % change due to interference to be clinically significant, it should be more than $1.96 \times (CV_a^2 + CV_w^2)^{1/2}$ [14] (CVa: analytical coefficient of variation, CVw: within-subject biological variation). The within-subject biological variation value of insulin is 21.1% and the analytical coefficient of variation is 10.6% [15]. According to the formula $1.96 \times (CV_a^2 + CV_w^2)^{1/2}$, a variation of more than 46% is expected. In our study, a change of 29.6% is observed at 200 HI at high insulin concentrations. It has been emphasized in studies that a 10% decrease in insulin concentrations due to hemolysis is considered clinically significant [2, 7, 10, 16]. In harmony, a percentage change of more than 10% due to hemolysis was obtained for both ACTH and insulin values in our study. Similarly, to our results, as the degree of hemolysis increased in previous studies, the insulin concentration decreased [3, 17, 18].

It was previously reported that NPM effectively prevented insulin loss in samples remaining at 30 and 37 °C without hemolysis [19]. Studies also show that hemolysis effect disappears when different IDE inhibitors, pcMPS, and diamides stabilize insulin, like NPM [3, 20]. However, the protective effect of NPM can vary among each hormone and different hormonal concentrations.

The fact that C-peptide is not affected by hemolysis may make it advantageous for the assessment of insulin
secretion [17]. However, there is one study showing that hemolysis interferes with the C-peptide [21]. IGF-1 was significantly affected by hemolysis, causing a significant decrease in its concentrations [22]. In our study, we showed that there was no decrease in C-peptide and IGF-1 concentrations due to hemolysis. The reason we could not detect interference in C-peptide and IGF-1 may be due to the insufficient degree of hemolysis. Another possibility is that proteases that degrade these peptides do not exist in erythrocytes.

In the present study, we demonstrated how proteolytic enzymes released from erythrocytes, due to hemolysis, degrade peptide hormones (ACTH and insulin) and decrease their levels. Consequently, to prevent the decrease in the concentrations of peptide structured hormones we treated samples with NPM, which is a protease inhibitor. We aimed to determine at which degree of hemolysis the interference started. Considering that it will offer a better perspective, we evaluated two different levels close to the clinical decision points. Upon examining the serum/plasma hemolysis degrees of the samples accepted to the laboratory, we thought the selected hemolysate indexes would be beneficial to consider in clinical practice.

In conclusion, with the addition of NPM, a protease inhibitor, the hemolysis effect, which is a source of pre-analytical error, was removed. This study may be crucial in accepting or rejecting samples by looking at the HI point where interference due to hemolysis begins. Furthermore, if supported by other studies, adding NPM to hemolyzed samples or producing tubes with NPM may be considered.

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Informed consent: None declared.

Ethical approval: The research related to human use has complied with all the relevant national regulations, institutional policies, and in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors’ Institutional Review Board or equivalent committee. Ankara University Faculty of Medicine Clinical Research Ethical Committee (03-128-18) issued approval the study. The study was done following the Declaration of Helsinki.

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