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Research article

# Effects of EDTA and Sodium Citrate on hormone measurements by fluorometric (FIA) and immunofluorometric (IFMA) methods

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## Abstract

**Background:** Measurements of hormonal concentrations by immunoassays using fluorescent tracer substance (Eu3+) are susceptible to the action of chemical agents that may cause alterations in its original structure. Our goal was to verify the effect of two types of anticoagulants in the hormone assays performed by fluorometric (FIA) or immunofluorometric (IFMA) methods.

**Methods:** Blood samples were obtained from 30 outpatients and were drawn in EDTA, sodium citrate, and serum separation Vacutainer®Blood Collection Tubes. Samples were analyzed in automatized equipment AutoDelfia™ (Perkin Elmer Brazil, Wallac, Finland) for the following hormones: Luteinizing hormone (LH), Follicle stimulating homone (FSH), prolactin (PRL), growth hormone (GH), Sex hormone binding globulin (SHBG), thyroid stimulating hormone (TSH), insulin, C peptide, total T3, total T4, free T4, estradiol, progesterone, testosterone, and cortisol. Statistical analysis was carried out by Kruskal-Wallis method and Dunn's test.

**Results:** No significant differences were seen between samples for LH, FSH, PRL and free T4. Results from GH, TSH, insulin, C peptide, SHBG, total T3, total T4, estradiol, testosterone, cortisol, and progesterone were significant different between serum and EDTA-treated samples groups. Differences were also identified between serum and sodium citrate-treated samples in the analysis for TSH, insulin, total T3, estradiol, testosterone and progesterone.

**Conclusions:** We conclude that the hormonal analysis carried through by FIA or IFMA are susceptible to the effects of anticoagulants in the biological material collected that vary depending on the type of assay.

## Background

Measurements of hormone concentrations in physiological fluids are used as biochemical indicators of endocrinological status and as therapy monitors [1]. Hormones are measured by immunoassays using different types of tracers, such as radioactive, enzymatic, chemiluminescent or fluorescent substances. The latter method has many advantages: it provides good resolution, is very accurate, has

long-lasting reagents, can be automated and is easy to perform [2]. However, it uses a fluorescent tracer substance (Eu3+) coupled to the antigen or antibody as a chelat [3], being susceptible to the action of chemical agents that may cause alterations in its original structure.

Currently, serum is the matrix of choice for hormone analysis through fluoro- or immunofluorometric assays [4]. EDTA is a powerful chelating agent and will bind to metallic ions that are a constituent of labels or act as cofactors for enzymatic activities [5].

In our routine analyses, we observed some elevated values whose results were not confirmed after dilution. The aim of this study was to verify the analytical variation among samples treated with EDTA and sodium citrate compared with serum samples, in hormone assays performed by fluorometric (FIA) and immunofluorometric (IFMA) methods.

## Materials and methods

Three different types of Vacutainer tubes were purchased from Becton Dickinson (Franklin Lanes, NJ, USA): (1) a serum separation tube which contained an inert barrier (gel) for cleaner separation of serum from coagulum during centrifugation (Vacutainer®Blood Collection Tubes, Hemograd SST), (2) a whole blood tube with liquid EDTA (Vacutainer®Blood Collection Tubes, K3 EDTA, 15%), (3) a whole blood tube with 0.129 mol/L buffered sodium citrate (Vacutainer®Blood Collection Tubes, 9NC, 3,8% sodium citrate). Blood samples were collected from 30 outpatients (11 men and 19 women; 19–61 years of age) after a minimum of 10 h fasting into EDTA, sodium citrate, and serum separation Vacutainer tubes. The serum or plasma was separated from the blood cells immediately after the blood was drawn by centrifugation at 2000 g for 5 min. Each sample was then analyzed in the automated machine AutoDelfia™ (Perkin Elmer Brazil, Wallac, Turku, Finland) for the following hormones, according to the manufacturer's instructions: LH spec, FSH, Prolactin (PRL), GH, SHBG, TSH Ultra, Insulin (INS) and C-Peptide (C-PEP) measured by IFMA method; Total T3 (TT3), Total T4 (TT4), free T4 (FT4), E2, Progesterone (PROG), Testosterone (TESTO), and Cortisol (F) measured by FIA method.

Statistical analysis was carried out by Kruskal-Wallis method followed by Dunn's test. The results from EDTA and citrate plasma were each compared to those measured in serum, and if there was a significant ($p < 0,05$) difference this was considered a significant effect of anticoagulant. All statistical analysis and plotting were performed using SigmaStat 2.0 software (Jandel Scientific, Chicago).

## Results

Tables 1 and 2 shows median and range of values obtained from hormonal measurements in serum, EDTA-treated and citrate-treated samples by IFMA and FIA assays respectively. The range of hormone concentrations varied among sample types (tables 1 and 2). No statistically significant differences were seen between EDTA-treated samples, sodium citrate-treated samples and serum samples in IFMA assays for LH spec ($p = 0.822$), FSH ($p= 0.653$), PRL ($p = 0.277$), and FIA assay for FT4 ($p = 0.208$), based on Kruskal-Wallis one way analysis of variance. Results from IFMA assays (Table 1) for SHBG, GH, TSH, INS, CPEP and FIA assays (Table 2) for TT3, TT4, E2, TESTO, F and PROG presented a statistically significant difference between serum and EDTA-treated sample groups. Compared to serum samples, EDTA-plasma samples rendered lower results for IFMA assays and higher values for FIA assays (Fig 1 and Fig 2). We also identified statistically significant differences between serum and sodium citrate-treated samples in the IFMA assays (Table 1) for TSH, INS, and FIA assays (Table 2) for TT3, TT4, E2, TESTO and PROG. We noted that F results of citrate-treated samples showed values slightly elevated when compared to serum samples results, but difference was not statistically significant (Table 2). Citrate-treated samples also rendered lower results for IFMA assays and higher values for FIA assays when compared to serum samples results (Table 1 and 2)

## Discussion

Blood collection for hormone analysis is an important factor in the quality of the final result, and if incorrectly carried out could produce problems such as physicians' interpretative errors, additional costs caused by patients' recall for extra collections and/or analysis replication for result confirmation.

EDTA can react with the labeled reagent and produce a chelat with the Eu3+ ion being eluted from the well. In FIA this effect is shown as an increased value, while in IFMA assays lower results are obtained. The manufacturer's instructions briefly describe which type of sample can be analyzed, warning users of the possibility of false results. However, in a routine laboratory that receives material in separated form, it is difficult to discriminate between serum and plasma.

In our study we observed a great influence of EDTA plasma on the competitive FIA assays with an inversely proportional relationship between the emitted signal and the concentration of the hormone. The EDTA and the citrate present in the sample extract the Eu3+ ions from the label through their chelating action, thus lowering the fluorescent signal and increasing final results. This fact explains the great rise of the values obtained in plasma assays of E2, TESTO, PROG, F, TT3 and TT4. In the non-competitive

**Table 1: Results of the hormonal measurements in serum, EDTA-treated and citrated-treated plasma (median and range) by IFMA assays. (p < 0.05)**

| | IFMA | | | | | |
|---|---|---|---|---|---|---|
| | SERUM | | EDTA | | CITRATE | |
| | median | range | median | range | median | range |
| **LH spec** IU/L | 5.9 | 0.07–54 | 5.6 | 0.06 -51 | 5.13 | 0.06 -49 |
| **FSH** IU/L | 6.8 | 0.02–119 | 6.55 | 0.01–108 | 5.83 | 0.01–94 |
| **PRL** ng/mL | 5.53 | 1.6–171 | 5.2 | 1.4–152 | 4.6 | 1.2–137 |
| **GH** ng/mL | 0.18 | 0.02–2.4 | 0.03*[a] | 0.01–4.7 | 0.11[a] | 0.01–8.2 |
| **INS** μIU/mL | 6.95 | 2.3–27 | < 4.8*# | <4.8 | 4.8* | 0.2–11 |
| **CPEP** ng/mL | 1.78 | 0.7–8.3 | 0.39*[a] | 0.2–1.7 | 1.13[a] | 0.4–4.9 |
| **SHBG** nmol/L | 47.1 | 14–367 | 28.6* | 8.3–315 | 37.5 | 11–336 |
| **TSH** μIU/mL | 1.46 | 0.03–100 | 0.03*[a] | 0.02–0.28 | 0.45*[a] | 0.01–53 |

range refers to minimum and maximum values * statistical significant difference between EDTA or citrate plasma results and serum (p < 0.05) [a] statistical significant difference between EDTA and citrate plasma results (p < 0.05) # Insulin in EDTA samples was undetectable.

**Table 2: Results of the hormonal measurements in serum, EDTA-treated and citrated-treated plasma (median and range) by FIA assays. (p < 0.05)**

| | FIA | | | | | |
|---|---|---|---|---|---|---|
| | SERUM | | EDTA | | CITRATE | |
| | median | range | median | range | median | range |
| **E2** pg/mL | 25.6 | 6.9–8280 | 2480*[a] | 949–10700 | 63.6*[a] | 41–8580 |
| **TESTO** ng/dL | 31.7 | 9.1–1060 | 687*[a] | 68–2800 | 202*[a] | 56–2460 |
| **PROG** ng/mL | 0.3 | 0.2–38 | 38*[a] | 23–38 | 0.7*[a] | 0.4–38 |
| **F** μg/dL | 11.7 | 6.1–37 | 145.6*[a] | 58–154 | 13.1[a] | 7.4–39 |
| **T3** ng/dL | 122 | 42–201 | >650*[a] | 204 – >650 | 209*[a] | 94 – >650 |
| **T4** μg/dL | 9.2 | 3.2 – 17 | 23.3*[a] | 11 – 23.3 | 10.2*[a] | 4.1 – 23.3 |
| **FT4** ng/dL | 0.91 | 0.43 – 1.62 | 0.91 | 0.43 – 1.96 | 0.99 | 1.46 – 2.19 |

range refers to minimum and maximum values * statistical significant difference between EDTA or citrate plasma results and serum (p < 0.05) [a] statistical significant difference between EDTA and citrate plasma results (p < 0.05)

IFMA assays, the relation between the signal and the concentration is directly proportional and the effect of anticoagulants is a reduction of the fluorescent signal with the consequent generation of falsely lower results. Four hormones did not present statistically significant differences (LH spec, FSH, PRL, and FT4), mostly analyzed by IFMA assays. Probably two incubations in the LH assays spec, FSH, and PRL explain the lesser influence of anticoagulants on these analyses. FT4 by FIA method, in which the effect was not observed, is the only assay that requires three incubations, and it is tempting to conclude that there is a lack of interference due to the shorter time of contact between reagents and/or to the dilution of the anticoagulant in the sample. In the FIA assays the effect is evidenced by elevated results that require many replications of the analyses, with sample dilutions. On the other hand, in IFMA assays this influence is not easily noticed, raising a question from the clinical standpoint.

Recently, Evans et al. examined the effect of three of the most commonly used anticoagulants and serum, as well as the effect with each of these collection methods of storing samples frozen, at 4°C or at 30°C for 24 and 120 hours, on 22 hormones measurements using radioimmu-

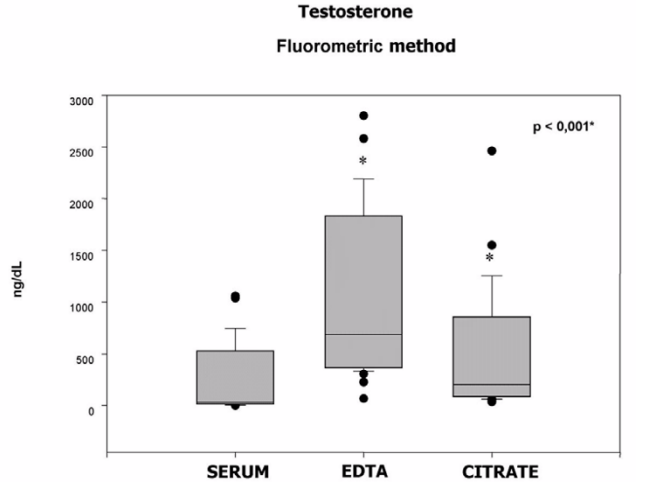


**Figure 1**
Effect of EDTA and citrate on the T assay using fluorometric method. Mean hormone concentrations (± SD) are represented by the box; medians are plotted as a line inside the box; error bars represents 10th and 90th percentiles; dots outside box are values that falls outside the 10th and 90th percentiles; significant ($^*p < 0.05$) differences between serum and EDTA or serum and citrate are indicated by an asterisk.

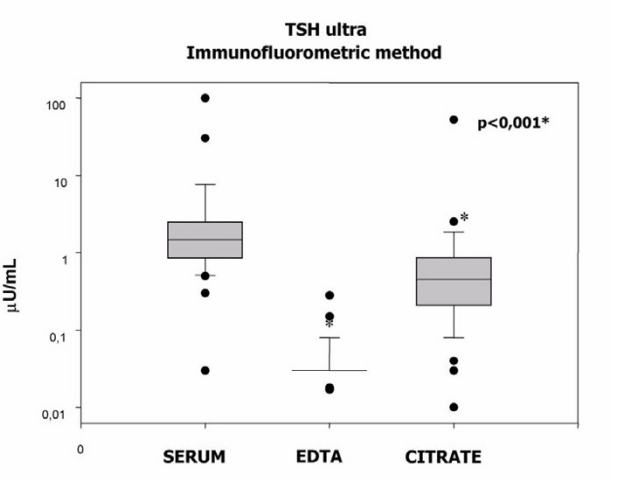


**Figure 2**
Effect of EDTA and citrate on the TSH assay using immunofluorometric method. Mean hormone concentrations (± SD) are represented by the box; medians are plotted as a line inside the box; error bars represents 10th and 90th percentiles; dots outside box are values that falls outside the 10th and 90th percentiles; significant ($^*p < 0.05$) differences between serum and EDTA or serum and citrate are indicated by an asterisk.

noassay, immunoradiometric assay or enzyme immunoassay. They found that when the different collection methods were compared to collection in EDTA, a significant effect was shown for several hormones. This suggests that samples sent to a laboratory should be collected into the appropriate anticoagulant for that laboratory's methods [6].

We conclude that the hormonal analyses carried out by FIA or IFMA are susceptible to the effect of anticoagulants in plasma and that the effect varies depending on the type of assay. The analyses must always be carried out in serum, free of metal-complexing anticoagulant, thus giving a more trustworthy result. Updating of the professionals involved with the collection of blood samples must be carried out, so that they are able to prevent interferences. Technicians should be aware of the possibility of interferences. This would both lower costs and provide better quality test results.

## Competing interests
None declared.

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## Pre-publication history
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