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Araştırma Makalesi / *Research Article*

Investigation Bisphenol A and Phthalate Levels in Idiopathic Hyperandrogenemia

İdiyopatik Hiperandrojenemili Hastalarda Bisfenol A ve Fitalat Düzeylerinin İncelenmesi

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ABSTRACT

Aim: Idiopathic hyperandrogenemia is defined as the disease in women with signs of hirsutism and increased serum androgen levels, a normal menstrual cycle, and normal ovarian morphology. In this study, it was aimed to examine the association of Bisphenol A and mono-ethylhexyl phthalate with this disease.

Material and Methods: A total of 91 individuals between the ages of 18–45 were included in the study. The patient group consisted of 43 women with high androgen levels who applied to the endocrine outpatient clinic with signs of hirsutism, and the control group consisted of 48 healthy women with no signs of hirsutism or any additional disease. Bisphenol A and mono-ethylhexyl phthalate were measured in urine by the liquid chromatography-mass spectrometry method.

Results: Urinary Bisphenol A levels were found to be significantly higher in the patient group compared to the control group (1.6 ng/ml; 0.55 ng/ml, p=0.035). Mono-ethylhexyl phthalate levels were found to be significantly higher in the control group compared to the patient group (1.81 ng/ml, 1.66 ng/ml, p=0.01). In logistic regression analysis, odds ratios were 1.91 (95% CI: 1.13–3.24, p<0.05) for BPA, 0.91 (95% CI: 0.81–1.01, p=0.09) for age, 18.74 (95% CI: 3.86–90.85, p<0.05) for fT3, and 1.06 (95% CI: 0.98–1.14, p=0.09) for prolactin.

Conclusion: Investigation of the effect of endocrine-disrupting chemicals in this group, which is less common in the etiology of hyperandrogenemia, made our study unique. The higher Bisphenol A urinary level in the patient group than in the control group suggests the effect of Bisphenol A in patients with idiopathic hyperandrogenemia. The higher level of mono-ethylhexyl phthalate in the control group suggests that more phthalate metabolites should be examined and more studies should be conducted.

Key Words: Bisphenol A, Phthalate, Idiopathic hyperandrogenemia, Endocrine disruptors

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ÖZET

Amaç: İdiyopatik hiperandrojenemi, kadınlarda hirsutizm belirtileri, artmış serum androjen düzeyleri, normal adet döngüsü ve normal yumurtalık morfolojisi ile seyreden bir hastalık olarak tanımlanır. Bu çalışmada, Bisfenol A ve mono-etilhekzil fitalat'ın bu hastalıkla ilişkisinin incelenmesi amaçlandı.

Materyal ve Metod: Araştırmaya 18-45 yaş arası toplam 91 birey dahil edildi. Hasta grubunu, endokrin polikliniğine hirsutizm bulgusu ile başvuran ve androjen düzeyi yüksek olan 43 kadın; kontrol grubunu ise hirsutizm bulgusu ve herhangi bir ek hastalığı olmayan 48 sağlıklı kadın oluşturdu. Bisfenol A ve mono-etilhekzil fitalat düzeyleri idrarda sıvı kromatografi-kütle spektrometri yöntemiyle ölçüldü.

Bulgular: Hasta grubunda idrar Bisfenol A düzeyleri, kontrol grubuna göre anlamlı olarak yüksek bulundu (1,6 ng/ml; 0,55 ng/ml, p=0,035). Mono-etilhekzil fitalat düzeyleri ise kontrol grubunda, hasta grubuna göre anlamlı olarak yüksek bulundu (1,81 ng/ml; 1,66 ng/ml, p=0,01). Lojistik regresyon analizinde odds oranları, BPA için 1,91 (%95 güven aralığı: 1,13-3,24; p<0,05), yaş için 0,91 (%95 güven aralığı: 0,81-1,01; p=0,09), fT3 için 18,74 (%95 güven aralığı: 3,86-90,85; p<0,05), prolaktin için 1,06 (%95 güven aralığı: 0,98-1,14; p=0,09) olarak bulundu.

Sonuç: Hiperandrojenemi etiyolojisinde daha az sıklıkla yer alan bu grupta, endokrin bozucu kimyasalların etkisinin incelenmesi çalışmamızı özgün kılmaktadır. Hasta grubunda Bisfenol A idrar düzeyinin kontrol grubuna göre yüksek olması, idiyopatik hiperandrojenemililerde Bisfenol A'nın etkisini düşündürmektedir. Kontrol grubunda ise mono-etilhekzil fitalat düzeylerinin daha yüksek bulunması, daha fazla ftalat metabolitinin incelenmesi ve daha fazla çalışma yapılması gerektiğini düşündürmektedir.

Anahtar Kelimeler: Bisfenol A, Fitalat, İdiyopatik hiperandrojenemi, Endokrin bozucular

INTRODUCTION

Hirsutism is a clinical finding that is characterised by the increased growth of terminal hair of a male type in women. Hirsutism may be the only symptom of androgen increase, or it may sometimes be accompanied by acne and androgenic alopecia. Excessive androgen elevation causes an increase in terminal hair (1). The distribution of hair is determined by Modified Ferriman Gallwey (MFG) scoring. Nine different parts of the body are scored between 0 and 4 and the total score is obtained. Cases with an MFG score of 8-15 are considered mild, cases between 16-25 are considered moderate, and cases above 25 are considered severe hirsutism. In Mediterranean coastal countries, an MFG score of 8 and above is considered hirsutism (2).

The most common cause of hirsutism is Polycystic Ovary Syndrome (PCOS). The most important disease group that should be considered in differential diagnosis is PCOS. The clinical presentation may include menstrual disorder, oligo-anovulation, hirsutism, infertility, insulin resistance, dyslipidemia and impaired glucose tolerance. Furthermore, acne, hirsutism, seborrhea, male pattern hair loss and acanthosis nigricans may result from hyperandrogenemia (3). This is followed by late-onset congenital adrenal hyperplasia (LOCAH) and idiopathic hirsutism/ hyperandrogenemia. Rarely, Cushing's syndrome, hyperprolactinemia, thyroid disorders, androgen-secreting tumors, some medications and smoking may increase hair growth (4).

Idiopathic hyperandrogenemia is defined as a group of patients with normal menstrual cycles and normal ovarian morphology, with hirsutism and increased serum androgen levels. In order to diagnose idiopathic hyperandrogenemia, other secondary causes of hirsutism must be excluded.

Endocrine Disrupting Chemicals

Endocrine Disrupting Chemicals (EDCs) are substances extensively used worldwide, particularly in the plastics industry, which has grown considerably in recent years. These chemicals have been recognized for their potential role in endocrine and metabolic

diseases. (5). The US Environmental Protection Agency (EPA) defines EDCs as exogenous substances that interfere with the natural hormones responsible for maintaining homeostasis and developmental processes (6). EDCs can exert both estrogenic and anti-estrogenic effects due to their complex steroidal structures (7). The intracellular signalling network is regarded as comprising both genomic and non-genomic pathways. The genomic pathway transports transcription factors in the target gene directly over nuclear receptors, while the non-genomic pathway transports membrane-bound ER-α and ER-β (estrogen receptors). ER-α receptors are generally found in the breast, uterus, ovarian theca cells, testis, and epididymis; ER-β receptors are found in the prostate, bladder, ovarian granulosa cells, adipose tissue, and colon. EDCs act on hormone-active organs (8,9).

Bisphenol A

Bisphenol A (BPA) is an organic compound consisting of two phenol rings. Bisphenol A is a widely prevalent organic compound found in various consumer products, including plastic food containers, baby bottles, and cans. BPA has been identified in a number of biological samples, including urine, blood, adipose tissue, breast milk and the placenta (10-14).

BPA binds to cell and nuclear receptors or stimulates these receptors through its BPA-mediated effect. There are many studies showing effects on estrogen receptor, androgen receptor, G-protein coupled estrogen receptor, insulin-like growth factor and estrogen-related gamma receptors (15-18). Studies on animals and humans have demonstrated BPA's negative effects on reproductive health (19,20).

After oral ingestion, BPA is metabolized in the liver by CYP2C18 (most commonly), CYP2C19 and CYP2C9. It is conjugated with glucuronic acid in the liver and its major metabolite, Bisphenol A glucuronide (BAPG), is formed. The minor metabolite Bisphenol-sulfate (BPAS) is formed. It has an average half-life of six hours. It is excreted in the urine together with its metabolites within 42 hours (21).

Phthalates

Phthalates are a group of synthetic esters of phthalic acid that differ in the length and branching of their alkyl side chains. Long-chain phthalates are used as plasticizers and short-chain phthalates are used as solvents. Phthalates used as plasticizers are used to increase the durability and flexibility of products. The ingestion of contaminated food, inhalation of phthalates in the air, and skin contact with products containing phthalates are the primary routes of entry for phthalates into the body (22,23).

Di-2-ethylhexyl phthalate (DEHP) is converted to mono-ethylhexyl phthalate (MEHP) metabolite by the catalysis of non-specific lipase enzymes, and then to many metabolites by side chain hydrolysis and cytochrome enzymes. The rate of conversion and excretion of phthalates to their metabolites make exposure assessment difficult. Exposure is usually assessed by measurement of phthalate metabolites in urine. However, analytical detection is possible only for some phthalate metabolites. Not all metabolites can be detected (24,25).

Study Objective

In this study, we aimed to measure BPA and MEHP levels in the urine of women with hirsutism and increased androgen levels and to compare them with the control group.

MATERIAL AND METHOD

Patient and Control group

The study population consisted of 43 female patients with idiopathic hirsutism, aged 18–45 years, who presented to the endocrine outpatient clinic of our hospital with complaints of increased body hair over the past year. At the outpatient clinic, a detailed

patient history, systemic physical examination, laboratory tests, and imaging studies were conducted to rule out potential diseases that could cause hair growth. Individuals were selected for inclusion in the study after thorough evaluation of all examination procedures and laboratory test results. Women with an MFG score of 8 or higher were included in the study. The patient group comprised female patients diagnosed with idiopathic hyperandrogenism.

The control group consisted of 48 healthy, age-matched women who had regular menstrual cycles, normal ovarian morphology, and no risk factors for hirsutism (MFG score below 8). These individuals presented to the same clinic for unrelated reasons. A comprehensive assessment, including laboratory and imaging tests similar to those performed on the patient group, was conducted to exclude any underlying medical conditions associated with increased hair growth. MFG scoring was performed consistently for all participants by the same physician.

Exclusion criteria

Individuals with PCOS, LOCAH, hyperprolactinemia, thyroid dysfunction, anti-androgenic drug therapy (e.g., oral contraceptives, hormone therapy), and smokers were excluded from the study.

Hyperprolactinemia was assessed after ruling out factors such as sleep, exercise, emotional and physical stress, breast or chest wall stimulation, coitus, and high-protein diets. A cut-off value of 25 ng/mL was used to define hyperprolactinemia (26). Similarly, 17-OH progesterone levels were measured to exclude a diagnosis of LOCAH, with a cut-off value of 2 ng/mL (27).

In patients with hirsutism, normal ovarian morphology, and regular menstrual cycles, a total testosterone level above 0.55 ng/mL was considered indicative of hyperandrogenemia (28). The diagnosis of PCOS was made based on the Rotterdam criteria, which require the presence of at least two out of three findings: oligo-anovulation (<6 menstrual periods per year), clinical or laboratory evidence of androgen elevation, or ultrasonographic evidence of polycystic ovary morphology (29).

Laboratory measurements and sample collection

In all cases, biochemical tests were performed on fasting serum samples collected at the same time. All blood samples were taken during the first three days of the menstrual cycle to standardize the hormonal tests. Biochemical tests were carried out using the spectrophotometric method with kits compatible with the Beckman Coulter 5800 (Brea, CA, USA) device. DHEA-S (Dehydroepiandrosterone sulfate), FSH (Follicle-stimulating Hormone), LH (Luteinizing Hormone), estradiol, progesterone, total testosterone, TSH (Thyroid Stimulating Hormone), fT3, fT4, prolactin, and insulin tests were performed with Access brand kits on the Beckman Coulter Unicel DxI 800 (Beckman Coulter, Miami, USA) device. The 17-OH progesterone test was conducted with the Snibe-Maglumi X3 (Shenzhen, China) model device and Snibe (China) brand kit. The androstenedione test was performed using the Immulite 2000 Xpi (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) by immunometric chemiluminescence. The free testosterone test was conducted using the ETI-Max 3000 (Diasorin, Italy) device and DiaMetra (Perugia, Italy) brand kit by the ELISA (Enzyme-Linked ImmunoSorbent Assay) method. The samples collected in gel and clot activator tubes (BD Vacutainer® SST™ II Plus Tubes) were centrifuged for 10 minutes at 2,000 x g within 30 minutes of collection. Urine samples from the cases were immediately transferred from plastic containers to glass tubes and stored at -20°C in glass tubes (BD Vacutainer® Serum Glass Tubes) for BPA and MEHP analysis.

Preparation of calibration standards

A mixed stock solution was prepared from concentrated 50 g BPA (CAS no: 80-05-7, Sigma-Aldrich, Taiwan) and 500 mg MEHP

(CAS no: 4376-20-9, Sigma-Aldrich, Taiwan) standard solution.

1. 50 µL of BPA and MEHP were taken from each bottle and diluted (1/1000) with 99.98% pure methanol (CAS no: 67-56-1, Carlo ERBA, France). A stock solution with a concentration of 10,000 ng/mL was prepared in a volume of 50 mL.
2. It was diluted again with distilled water (1/10).
3. A stock solution with a concentration of 100 ng/mL was prepared by diluting once again with pure methanol (1/10).

By making serial dilutions with methanol; Six-level standard solutions were prepared at concentrations of 100, 25, 10, 5, 1 and 0.25 ng/mL, respectively.

Each prepared standard solution was mixed equally with Acetonitrile precipitator (Cas no: 75-05-8, Carlo ERBA, France) and vortexed for 1 minute. It was centrifuged for 10 minutes at 4000 rpm in a NUVE NF 800 (Ankara, TR) brand centrifuge device, and the supernatants were placed in separate glass vials and made ready for study.

Sample preparation and analysis process

Urine samples stored frozen at -20°C were kept at room temperature until completely thawed. 300 µL urine sample from the patient and control groups and 300 µL acetonitrile were mixed and centrifuged (10 minutes at 4000 rpm). Supernatants were taken into separate glass vials.

Mobile phase solution A contained 0.1% acetic acid and water, and mobile phase solution B contained 0.1% acetic acid and acetonitrile. The column had a size of 100 mm x 2.1 mm, particle width of 2.7 µm (Lot No 220113.1, ReproShel Phenyl-Hexyl, Dr Maisch GmbH, Germany). Column temperature was 40 °C. The flow rate was set to 0.4 mL/min. The total run time for each sample was 12 minutes. The analyzer temperature was kept between 8-10 °C, the room temperature was at 26 °C. The injection volume was optimized to 20 µL.

Analytes were scanned by LC/MS-MS in ESI negative MRM (multiple reaction monitoring) mode on an AB SCIEX QTRAP 4500 (AB Sciex Technologies, Framingham, MA, USA) device.

Statistical evaluation

Descriptive statistics for this study are presented as the number of individuals (n), mean, and standard deviation (SD) from the dataset. The distribution of variables was examined using box plots, and extreme values that did not conform to the distribution were identified. The normality of the data was assessed with the Shapiro-Wilk test. For comparisons of means between two groups, the Independent Sample T-test was applied if the data followed a normal distribution; otherwise, the Mann-Whitney U test was used.

To analyze continuous variables, Spearman correlation was employed when the assumption of normal distribution was not met. The logistic regression model included variables such as age, fT3, and prolactin in both the patient and control groups. This model assessed the effect of BPA on idiopathic hyperandrogenism while controlling for these variables.

In all comparisons, a p-value of <0.05 was considered statistically significant. Data analysis was performed using IBM SPSS Statistics 25 (IBM Corp., Armonk, New York, USA).

RESULTS

Device images of BPA and MEHP results for four patients are shown below. The mass values of the precursor ion, retention times, and scanned areas are presented in Figure 1. Within 12 minutes, the BPA peak was detected at 4 minutes and 56 seconds, while the MEHP peak was detected at 5 minutes and 80 seconds. The scanned area represents the concentration of the measured analyte (Figure 1) and was calculated by a single experienced laboratory technician.

Extreme values of variables not suitable for distribution were identified using box plots. A total of 13 potential outliers were found for BPA and MEHP measurements in the control and patient groups. However, one participant from the patient group (67) was identified as an extreme outlier for BPA measurement. This participant was excluded from the dataset as their inclusion would introduce bias into the analyses (Figure 2).

Four laboratory results indicative of hyperandrogenemia—total testosterone, free testosterone, androstenedione, and DHEA-S—were clinically and statistically significantly higher in the patient group compared to the control group. BPA levels were higher in the patient group than in the control group (95% CI; mean: 1.630 [2.665–0.655], 0.556 [0.803–0.308] ng/mL, p = 0.035). Conversely, MEHP levels were higher in the control group than in the patient group (95% CI; mean: 1.817 [1.920–1.715], 1.665 [1.764–1.570] ng/mL, p = 0.011) (Table 1).

The table below compares BPA and MEHP levels between the control and patient groups, along with laboratory test results indicative of hyperandrogenemia. A significant negative correlation was observed between MEHP and androstenedione in the patient group ($r = -0.302$, $p < 0.05$) (Table 2).

In logistic regression analysis, the odds ratios were as follows: 1.91 (95% CI: 1.13–3.24, $p < 0.05$) for BPA, 0.91 (95% CI: 0.81–1.01, $p = 0.09$) for age, 18.74 (95% CI: 3.86–90.85, $p < 0.05$) for fT3, and 1.06 (95% CI: 0.98–1.14, $p = 0.09$) for prolactin (Table 3).

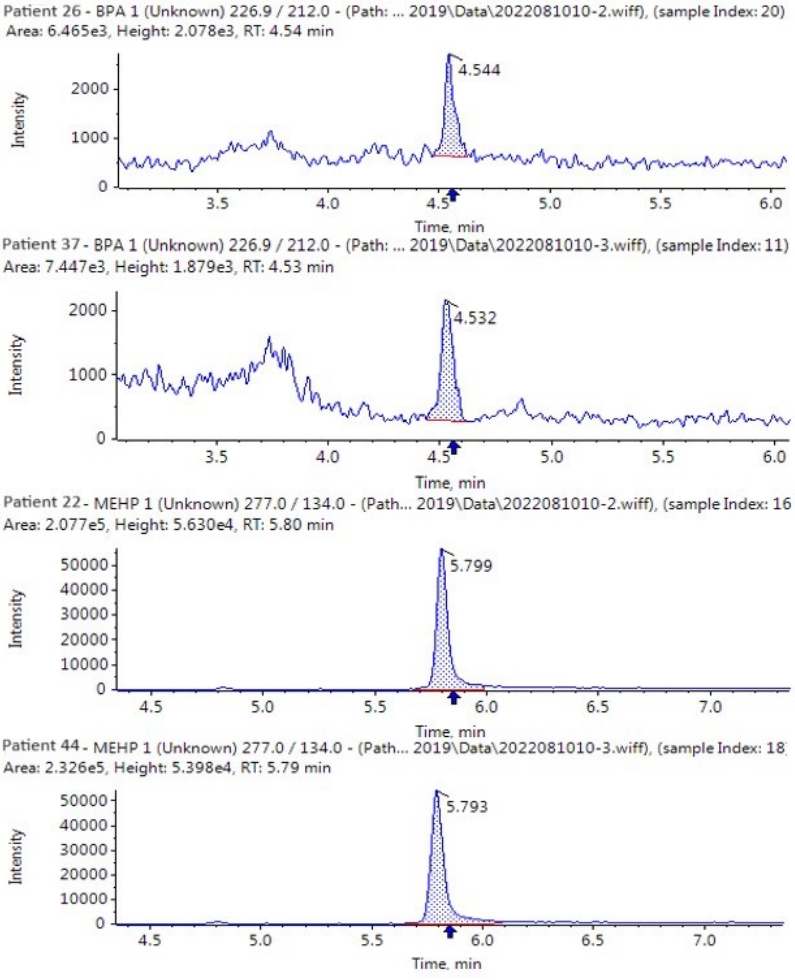

Figure 1. Device images of BPA and MEHP results for 4 patients

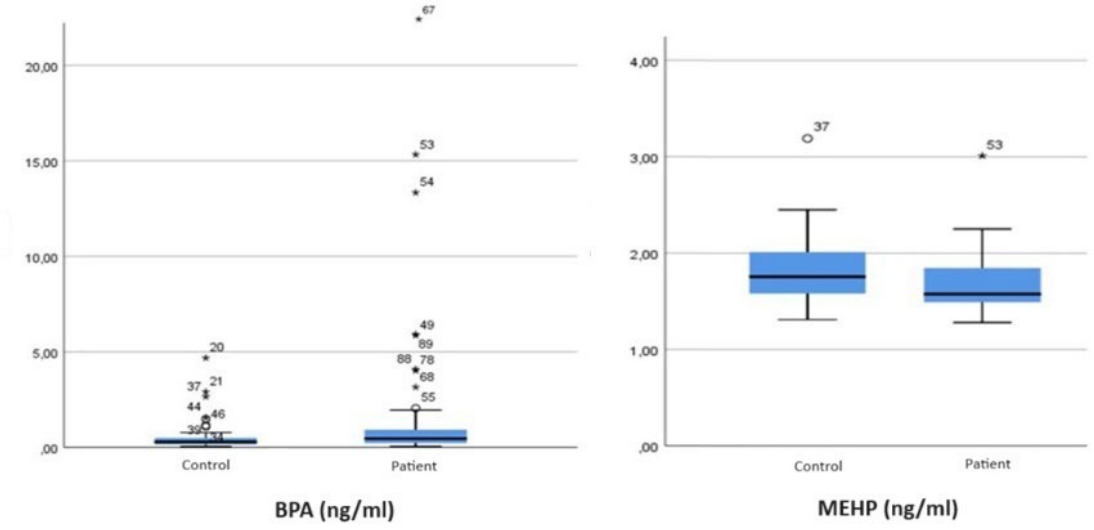

Figure 1. BPA and MEHP measurements in the control and patient groups

Table 1. Comparison of clinical and laboratory findings of patient and control groups
Tablo 1. Hasta ve kontrol gruplarının klinik ve laboratuvar bulgularının karşılaştırılması

		n	Mean	SD	p
Modified Ferriman Gallwey	C	48	3,90	2,136	0,000*
	P	43	17,98	5,930	
Fasting blood glucose (mmol/L)	C	48	4,95	0,49	0,881
	P	43	4,93	0,55	
Total Testosterone (pmol/L)	C	48	1770,9	551,9	0,000*
	P	43	2749,3	719,4	
Free Testosterone (pmol/L)	C	48	2,80	1,62	0,000*
	P	43	8,45	5,20	
Androstenadione (nmol/L)	C	48	5,06	2,86	0,000*
	P	43	12,44	5,75	
DHEA-S (μmol/L)	C	48	5,72	2,57	0,000*
	P	43	8,53	3,84	
TSH (mU/mL)	C	48	1,930	0,919	0,171
	P	43	2,180	0,970	
fT3 (pmol/L)	C	48	5,31	0,57	0,000*
	P	43	5,93	0,67	
fT4 (pmol/L)	C	48	11,32	1,42	0,067
	P	43	10,81	2,06	
Prolactin (μg/L)	C	48	13,341	6,500	0,040*
	P	43	16,769	12,055	
BPA (ng/mL)	C	48	0,556	0,851	0,035*
	P	43	1,630	3,191	
MEHP (ng/mL)	C	48	1,817	0,352	0,011*
	P	43	1,665	0,307	

n: number of participants; SD: standart deviation; *p<0,05; C: Control P: Patient

Table 2. Comparison of BPA and MEHP with laboratory findings indicating hyperandrogenemia

Tablo 2. BPA ve MEHP'nin hiperandrojenemiyi gösteren laboratuvar bulgularıyla karşılaştırılması

		Control		Patient	
		BPA	**MEHP**	**BPA**	**MEHP**
MEHP (ng/ml)	r	0,123	1,000	0,239	1,000
	p	0,404	-	0,122	-
Total Testosterone (pmol/L)	r	0,183	-0,080	0,093	-0,145
	p	0,212	0,591	0,553	0,352
Free Testosterone (pmol/L)	r	0,253	0,080	0,160	-0,198
	p	0,083	0,590	0,306	0,204
Androstenadione (nmol/L)	r	0,234	0,037	0,060	-0,302
	p	0,110	0,803	0,704	0,049*
DHEA-S (μmol/L)	r	0,050	-0,069	0,221	0,061
	p	0,737	0,642	0,154	0,696

*p<0,05 r = Spearman's rank correlation coefficient (Rho)

Table 3. The results of logistic regression analysis

Tablo 3. Lojistik regresyon analizinin sonuçları

	OR	**%95 CI**	***P***
BPA	1.91	1.13-3.24	< 0.05
Age	0.91	0.81-1.01	0.09
fT3	18.74	3.86-90.85	< 0.05
Prolactin	1.06	0.98-1.14	0.09

OD=Odds Ratio, CI=Confidence Interva

DISCUSSION

The diagnosis of idiopathic hyperandrogenism should be made by carefully performing a differential diagnosis using clinical experience, laboratory tests, and imaging studies. Although the etiology of the disease is not fully understood, we believe that environmental factors may play a role. Several studies have indicated that bisphenol and phthalate metabolites may be associated with the development of other endocrine disorders. While BPA and phthalate derivatives have been compared with many diseases in the literature, the lack of a study investigating the relationship between idiopathic hyperandrogenism and these endocrine disruptors makes our study unique.

In chromatographic methods, the pre-analytical steps of sample preparation are crucial. During the pre-analytical phase, storing the samples under appropriate conditions until the day of analysis, avoiding plastic contamination, and ensuring that the tubes used are free of plastic content are important for accurately determining bisphenols and phthalates. Markham et al. added acetonitrile and methanol into standard blood collection tubes and observed BPA exposure at room temperature for 1, 8, and 24 hours. BPA was detected as contamination in the extraction solvents (0.1 to 10 ng/mL) (30). In our study, there was no process of keeping the samples in plastic tubes during the collection phase. However, to evaluate potential plastic contamination from the injection line and pipette tips used during sample preparation, glass vials containing only methanol were included for blind analysis. No signals were detected for the analytes.

Previous studies have noted that liquid-liquid extraction and solid-phase extraction

processes are time-consuming and involve the use of large amounts of organic solvents. Xiao et al. obtained a mobile phase mixture of 70% acetonitrile and 30% water for optimal separation of BPA and interfering substances, using this mixture as an elution phase (31). In our study, we observed that the optimum analyte could be obtained using the acetonitrile precipitation method. Similar to Xiao et al., mobile phase solution A used in our study contained 0.1% acetic acid and water, while mobile phase solution B contained 0.1% acetic acid and acetonitrile. Chen J. et al. demonstrated 13 phthalate metabolites, including MEHP, in urine using the ultra-performance liquid chromatography tandem mass spectrometry (UPLC/MS-MS) method (32). Similarly, in our study, precipitation with acetonitrile was performed after incubation with beta-glucuronidase enzyme, and the eluate was obtained.

Ying Hu et al., in a meta-analysis of nine studies involving 493 PCOS patients and 440 controls, demonstrated elevated serum BPA levels in PCOS patients using HPLC and ELISA methods (standardized mean difference (SMD): 2.437 ng/mL, 95% CI (1.265, 3.609), $p < 0.001$) (33). In our study, BPA levels (95% CI, mean: 1.630 (2.665–0.655), 0.556 (0.803–0.308) ng/mL, $p = 0.035$) were found to be higher in the patient group using the LC/MS-MS method.

Konieczna A. et al. compared serum BPA levels in 106 PCOS patients and 80 controls using the HPLC method. Women with PCOS had significantly higher BPA levels than the control group (geometric mean and (95% CI): 0.202 ng/mL (0.150; 0.255) vs. 0.154 ng/mL (0.106; 0.201), $p = 0.035$). BPA levels were positively correlated with serum total testosterone ($r = 0.285$, $p = 0.004$) and the free androgen index ($r = 0.196$, $p = 0.049$) (34). In our study, we could not evaluate the free androgen index because we did not measure sex hormone-binding globulin. However, similar to Konieczna A. et al., a positive correlation was observed between BPA levels and total testosterone levels ($r = 0.093$, $p = 0.553$).

Takeuchi et al. compared BPA levels using the ELISA method in 19 PCOS patients and 26 women with regular menstruation. They found that BPA levels were positively correlated with total testosterone ($r = 0.39$, $p < 0.001$), androstenedione ($r = 0.68$, $p < 0.001$), DHEA-S ($r = 0.51$, $p < 0.001$), and free testosterone ($r = 0.50$, $p < 0.001$) (35). In our study, positive correlations were observed between BPA levels in the patient group and total testosterone ($r = 0.093$, $p = 0.553$), free testosterone ($r = 0.160$, $p = 0.306$), androstenedione ($r = 0.060$, $p = 0.704$), and DHEA-S ($r = 0.221$, $p = 0.154$).

Chou et al. evaluated urinary concentrations of mono-n-butyl phthalate (MnBP), mono(2-ethylhexyl) phthalate, monobenzyl phthalate, mono(2-ethyl-5-oxo-hexyl) phthalate, and mono(2-ethyl-5-hydroxyhexyl) phthalate in 123 women with endometriosis and 78 controls. They found that only MnBP was associated with endometriosis (95% CI; mean: 1.89 (1.05–3.39) μg/g) (36). Chen J. et al. evaluated urinary phthalate exposure in 220 individuals with thyroid nodules and 220 controls. MEHP levels were found to be higher in the patient group than in the control group (mean: 2.87 vs. 2.66 μg/g, $p = 0.985$) (32). Akın L. et al. compared serum levels of DEHP and MEHP in 63 adolescent PCOS patients and 61 controls, with higher DEHP and MEHP levels observed in the control group (37). Similarly, in our study, MEHP levels were higher in the control group than in the patient group (95% CI, mean: 1.817 (1.920–1.715), 1.665 (1.764–1.570) ng/mL, $p = 0.011$). Since MEHP levels alone do not indicate total phthalate exposure, we believe further studies are needed to include other phthalate derivatives.

Prolactin ($p = 0.040$) and fT3 ($p < 0.001$) levels were statistically significantly higher in the patient group than in the control group. However, since all individuals were within reference ranges for these parameters, they were not considered among the etiological

causes of hirsutism. Logistic regression analysis highlighted BPA as a potential risk factor for idiopathic hyperandrogenism. The findings that prolactin and fT3 levels were statistically significantly higher in the patient group suggest their limited effect on hirsutism.

A limitation of this study is that the level of dihydrotestosterone (DHT), a potent peripherally acting hormone, was not measured. Another limitation is the sample size. Differential diagnosis of idiopathic hyperandrogenism requires considerable time and collaboration with clinicians. Recent experimental studies have shown that BPA disrupts DHT-induced androgen receptor dimerization and negatively affects androgen receptor function. Evidence from laboratory and human studies suggests that BPA has anti-androgenic activity by binding to androgen receptors, adversely affecting cell development and function (38,39).

Our results suggest that environmental factors should be considered in the differential diagnosis of hirsutism and that the possible endocrine-disrupting effects of these chemicals should be further investigated. In conclusion, the association of BPA and MEHP with hirsutism is an important research area that should be supported by further clinical and epidemiological studies. This study provides a foundation for understanding the effects of environmental factors on hormonal imbalances.

Ethic Approval

Approval was obtained from the ethics committee of our institution with the decision numbered 17 and dated February 03, 2022.

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Conflict of interest

None

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Araştırma Makalesi / *Research Article*

Comparison of Urine Drug Abuse Testing Analyzed in Glass and Plastic Tubes

Cam ve Plastik Tüplerde İdrarda Uyuşturucu Bağımlılığı Testlerinin Karşılaştırılması

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ABSTRACT

Objectives: Drug abuse tests are applied to determine drug use to combat disease, crime, or substance abuse. We aimed to investigate whether cheaper and more practical plastic tubes can be an alternative to glass tubes for drug abuse analysis in urine.

Material and Methods: Paired fresh urine samples from 80 volunteers were collected into glass and plastic tubes, and drug abuse tests were analyzed in parallel after specimen validity tests. Amphetamines (AMP), benzodiazepines (BNZ), cocaine (COC), opiates (OPI), and cannabinoids (THC) were measured semi-quantitatively using the immunoassay method with the Roche Cobas c 501. Cut-off values for positivity were taken as AMP > 500 μg/L, BNZ > 300 μg/L, COC > 150 μg/L, OPI > 2000 μg/L, and THC > 50 μg/L. To investigate the effect of time, 32 of the samples were stored at room temperature for 4 hours without any preservative and reanalyzed. The SPSS 25.0 program was used to analyze the data.

Results: There were no significant differences between glass and plastic tubes in AMP, BNZ, COC, OPI, and THC concentrations. A statistically significant difference was found for OPI ($p = 0.005$), but the difference in OPI levels did not change the clinical decision. There were high correlations between all results for the same analyte measurements in both tubes ($p = 0.0001$). Additionally, 4-hour storage at room temperature did not cause degradation or adsorption of any drug.

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Conclusion: As a result of this study, it can be concluded that plastic tubes can be used instead of glass tubes in the analysis of drugs of abuse, with no significant change in substance concentrations after storage for four hours at room temperature.

Key Words: Urine, Drug abuse testing, Substance abuse

ÖZET

Amaç: Madde bağımlılığı testleri tıbbi, adli vakalarda ve denetimli serbestlik hastalarında uyuşturucu kullanımının tespiti amacıyla kullanılmaktadır. Biz bu çalışmada idrarda madde analizinde ucuz ve pratik olan plastik tüplerin cam tüplere alternatif olup olamayacağını araştırmayı amaçladık.

Gereç ve Yöntem: 80 yetişkin gönüllüden alınan taze idrar örnekleri cam ve plastik tüplere aktarıldı ve örneklerde idrar bütünlük testleri çalışılarak örneklerin uygunlukları değerlendirildi. Ardından idrarda amfetaminler (AMP), benzodiazepinler (BNZ), kokain (COC), opiatlar (OPI) ve kannabinoidlerin (THC) düzeyi Roche Cobas c 501'de immünoanaliz yöntemiyle yarı kantitatif olarak ölçüldü. Testin pozitifliği için kesim değerleri; AMP> 500μg/L, BNZ> 300μg/L, COC> 150μg/L, OPI> 2000μg/L ve THC> 50μg/L olarak alındı. İdrar örneklerinin bekletilmesinin test sonucuna etkisini araştırmak için, numunelerden 32 tanesi herhangi bir koruyucu madde olmadan dört saat bekletildi ve tekrar analiz edildi. Verilerin analizinde SPSS 25.0 programı kullanıldı.

Bulgular: AMP, BNZ, COC, OPI ve THC konsantrasyonlarında cam ve plastik tüpler arasında anlamlı bir fark yoktu. OPI açısından istatistiksel olarak anlamlı fark bulundu (p= 0.005) ancak OPI düzeylerindeki değişiklik klinik kararı (pozitiflik) değiştirmedi. Her iki tüpte de aynı analit ölçümüne ilişkin tüm sonuçlar arasında anlamlı yüksek korelasyon vardı (p= 0.0001). Bekletilen idrar numunelerinde (n= 32) sadece BNZ plastik tüpte istatistiksel olarak anlamlı yüksek bulundu (p= 0.037); ancak bu farklılıklar örneklerin pozitifliğini veya negatifliğini değiştirmedi ve klinik olarak anlamsızdı. Tüm testler için tüp türleri ve süre açısından örnekler karşılaştırıldığında anlamlı korelasyonlar mevcuttu (p <0.001).

Sonuç: Bu çalışmanın sonucunda, kötüye kullanılan ilaçların analizinde cam tüpler yerine plastik tüplerin kullanılabileceği, oda sıcaklığında dört saat saklandıktan sonra madde konsantrasyonlarında anlamlı bir değişimin olmadığı sonucu çıkarılabilir.

Anahtar kelimeler: İdrar, Madde analizleri, Yasadışı madde analizleri

INTRODUCTION

Drug abuse is considered one of the major preventable public health and safety problems in the world. The fight against drugs is among the priority goals of countries, and determining substance use through drug screening is one of the most important elements in this fight (1). Drug screening through urinalysis is the most suitable and widely accepted tool for rapidly detecting potential drug use (2). Immunochemical methods are frequently used in medical laboratories for urine drug screening. Urine drug screening by immunoassay in routine laboratories is an automated, simple, rapid, semi-quantitative, and cost-effective analysis. A single urine sample can be used to analyze all desired stimulants and drugs by immunoassays.

The substances that test positive in drug screening analysis are then subjected to confirmatory analysis upon administrative or judicial request. The confirmatory analysis is performed using gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS/MS), providing quantitative detection with the same urine specimen (3,4). The urine specimen collection is simple, non-invasive, and allows for a wide detection window for most drugs and/or drug metabolites (2). The sample collection container must be suitable for the specimen to ensure it does not affect analytical test results.

Recently, plastic collection tubes have started to replace glass tubes in many laboratories. These disposable plastic tubes are cheaper than glass tubes and are also suitable for storage at low temperatures and transportation. However, depending on the physicochemical properties, some drug analytes tend to adhere to plastic surfaces (5). Plastic tubes have been shown to

influence the measured concentrations or stabilities of various therapeutic drugs and peptide hormones in the blood (6,7,8).

This study aims to investigate the impact of urine collection tubes on drug abuse tests and evaluate whether plastic tubes can serve as an alternative to glass tubes.

METHODS

The urine drug analysis of 80 drug abusers, aged between 18-65 years, who applied to the Alcohol and Substance Abuse Treatment and Research Centre (AMATEM), affiliated with the Psychiatry Clinic of the University Hospital, was included in this study. Urine samples were collected according to the quality and safety requirements outlined in the latest regulation of the Department of Medical Laboratory Services, Ministry of Health in Turkey (9). To address the problem of tampering with the urine specimens, the fresh urine samples were collected under observation, separated into glass and plastic tubes (Vacusera Urine Tube No Additive) simultaneously, and transferred to the laboratory for analysis.

We used polypropylene tubes, which are a type of plastic commonly used in laboratories due to their low adsorption properties, chemical resistance, and durability. Drug abuse analysis began after the specimen integrity tests, such as creatinine levels, specific gravity, and pH values, were used to detect substitution, adulteration, or dilution (10). To investigate the effect of time, 32 samples were stored at room temperature for 4 hours without any preservative and reanalyzed.

The levels of amphetamines (AMP), benzodiazepines (BNZ), cocaine or metabolites (COC), opiates (OPI), and cannabinoids (THC) in urine were measured using Roche Cobas c 501 auto analyzers (Roche Diagnostics GmbH, Mannheim, Germany) with the kinetic interaction of microparticles in solution (KIMS) method. This is an in vitro diagnostic test for semi-quantitative and qualitative measurements. The cut-off values for positivity were defined as AMP > 500 µg/L, BNZ > 300 µg/L, COC > 150 µg/L, OPI > 2000 µg/L, and THC > 50 µg/L (9).

The daily internal quality control results (negative and positive levels according to the cut-off values) were acceptable for any drug test (11). The external quality assessment (EQA) was also acceptable, with positive or negative results matching the target positive or negative results of each drug test in external quality control material (Oneworld Accuracy EQA Program, Vancouver, Canada). Written consent was routinely obtained from all participants. The study was approved by the Research Ethics Commission of the University (Approval No. 60116787-020/2372, Date: 25.12.2018), with respect to the ethical standards of the Declaration of Helsinki.

Statistical Analysis

IBM SPSS Statistics 22 (SPSS Inc., Chicago, Ill, USA) program was used for statistical analysis. The suitability of the parameters to the normal distribution was evaluated by Kolmogorov-Smirnov and Shapiro-Wilks tests. In addition to descriptive statistical methods (minimum, maximum, mean, standard deviation, median frequency), Student's t-test was used for comparisons of normally distributed parameters between two groups, and Mann Whitney U test was used for comparisons of non-normally distributed parameters between two groups. The Chi-Square test and Fisher's Exact Chi-Square test were used to compare qualitative data. A p-value less than 0.05 was set as the statistical significance level. Bland–Altman analysis and correlation coefficients were used to evaluate the interchangeability between plastic and glass tube (as a reference) results.

RESULTS

The temperatures of the samples were between 33–37 °C. The urine integrity tests were performed, and all results were within acceptable ranges [(The acceptable ranges:

pH: 3–11, specific gravity: 1003–1035, creatinine: 20–200 mg/dL, nitrite: negative (Cut-off: 500 mg/L)] (9,12,13). Next, five different drug abuse tests were performed on 80 paired samples drawn into glass and plastic tubes.

Among the 80 patients' urine samples, positive results were as follows: BNZ (n=6), COC (n=1), AMP (n=5), OPI (n=9), and THC (n=5) in both glass and plastic tubes. In the 32 samples stored at room temperature for 4 hours, the positivity or negativity of results was not affected.

Paired t-tests of 80 samples showed no statistically significant differences between glass and plastic tubes for AMP, COC, BNZ, and THC. However, a statistically significant difference (p= 0.005) was found for OPI (Table 1). For stored samples (n= 32), only BNZ showed a statistically significant difference (p= 0.037), with BNZ concentrations being higher in plastic tubes (Table 2). Nonetheless, these differences did not change the positivity or negativity of the samples and were determined to be clinically insignificant.

There was no statistically significant difference between the 0-minute and 4-hour measurements in either plastic or glass tubes at room temperature ($p > 0.05$). Both types of tubes showed similar results for all analytes, and the 4-hour storage duration at room temperature did not significantly affect the test outcomes (Table 3).

Spearman correlation coefficients (r) comparing tube types and storage time for all tests showed statistically significant agreement ($p< 0.001$) with strong correlations. Intraclass correlation coefficients (ICC) for tube comparisons are presented in Table 4. Bland-Altman plots for all tests are provided in Figure 1.

Table 1. Comparison data of glass vs. plastic collection tubes on test results

Table 1. Cam ve plastik toplama tüplerinin test sonuçları üzerindeki etkilerinin karşılaştırılması.

	Glass (n= 80)		Plastic (n= 80)		
Tests (µg/L)	**Median**	**Mean**	**Median**	**Mean**	**p-value**
Benzodiazepines (BNZ)	38 (0 - 3472)	165.08 ± 503.58	43.5 (0 - 3460)	166.99 ± 501.14	0.304
Cocaine (COC)	13 (0 - 1301)	28.98 ± 144.47	14 (0 - 1332)	29.41 ± 147.87	0.961
Amphetamines (AMP)	66.5 (0 - 552)	98.03 ± 118.1	69 (0 - 561)	100.54 ± 117.11	0.247
Opiates (OPI)	29.5 (0 - 239184)	3306 ± 26721.71	21.5 (0 - 211463)	2955.65 ± 23625.39	0.005*
Cannabinoids (THC)	4.5 (0 - 366)	15.51 ± 47.92	4 (0 - 350)	15.29 ± 46.83	0.625

Statistically significant results (<0.05) are indicated with an asterisk (*).

Table 2. Comparison data of glass vs. plastic collection tubes after stored for 4 hours at room temperature on test results

Table 2. Cam ve plastik toplama tüplerinin, oda sıcaklığında 4 saat saklandıktan sonraki test sonuçları üzerindeki etkilerinin karşılaştırılması

	Glass (n= 32)		Plastic (n= 32)		
Tests (µg/L)	**Median**	**Mean**	**Median**	**Mean**	**p-value**
Benzodiazepines (BNZ)	8 (0 - 1980)	165.75 ± 464.09	15.5 (0 - 1994)	174.5 ± 463.68	0.037*
Cocaine (COC)	12.5 (0 - 34)	11.63 ± 9.64	7 (0 - 32)	10.81 ± 10.86	0.513
Amphetamines (AMP)	67 (5 - 527)	99.44 ± 122.33	67 (0 - 520)	102.66 ± 118.69	0.61
Opiates (OPI)	9.5 (0 - 5317)	500.94 ± 1328.61	20 (0 - 5289)	502.47 ± 1320.97	0.603
Cannabinoids (THC)	5.5 (0 - 179)	12.91 ± 31.99	6 (0 - 155)	12.63 ± 27.88	0.359

Statistically significant results (<0.05) are indicated with an asterisk (*)

Table 3. Comparison of 0-minute and 4-hour Measurements in Plastic and Glass Tubes at Room Temperature
Table 3. Plastik ve cam tüplerde oda sıcaklığında 0. dakika ve 4. saat ölçümlerinin karşılaştırılması

	Glass (n:32)				
	0-minute		4 hours at RT		
Tests (µg/L)	**Median**	**Mean**	**Median**	**Mean**	**p**
Benzodiazepines (BNZ)	10 (0 - 1974)	165.08 ± 490.48	8 (0 - 1980)	165.75 ± 464.09	0.966
Cocaine (COC)	12.4 (0 - 39)	12 ± 10.26	12.5 (0 - 34)	11.63 ± 9.64	0.871
Amphetamines (AMP)	66.5 (0 - 552)	100.03 ± 127.64	67 (5 - 527)	99.44 ± 122.33	0.794
Opiates (OPI)	10.5 (0 - 5327)	489.42 ± 955.43	9.5 (0 - 5317)	500.94 ± 1328.61	0.853
Cannabinoids (THC)	4.5 (0 - 163)	12.51 ± 42.92	5.5 (0 - 179)	12.91 ± 31.99	0.787
	Plastic (n:32)				
Benzodiazepines (BNZ)	24 (0 - 1951)	175 ± 476.94	15.5 (0 - 1994)	174.5 ± 463.68	0.995
Cocaine (COC)	10 (0 - 39)	11.56 ± 8.54	7 (0 - 32)	10.81 ± 10.86	0.85
Amphetamines (AMP)	69 (0 - 561)	108.54 ± 117.11	67 (0 - 520)	102.66 ± 118.69	0.868
Opiates (OPI)	21.5 (0 - 5475)	515 ± 1419.09	20 (0 - 5289)	502.47 ± 1320.97	0.992
Cannabinoids (THC)	4 (0 - 167)	11.80 ± 19.63	6 (0 - 155)	12.63 ± 27.88	0.897

Table 4. Intraclass correlation coefficients of drug abuse tests
Table 4. Uyuşturucu madde testlerinin sınıf içi korelasyon katsayıları

	The intraclass correlation for the 5 tests in 2 tubes at each time interval		
	Intraclass correlation coefficients (ICC)	**95% confidence interval (95% CI)**	**p-value**
BZN plastic-glass	1	1.000 - 1.000	<0.001*
4 hours at RT	0.999	0.998 - 0.999	<0.001*
AMP plastic-glass	0.994	0.991 - 0.996	<0.001*
4 hours at RT	0.993	0.986 - 0.997	<0.001*
COC plastic-glass	0.999	0.999 - 1.000	<0.001*
4 hours at RT	0.873	0.742 - 0.937	<0.001*
OPI plastic-glass	0.996	0.994 - 0.981	<0.001*
4 hours at RT	1	1.000 - 1.000	<0.001*
THC plastic-glass	0.999	0.998 - 0.999	<0.001*
4 hours at RT	0.994	0.987 - 0.997	<0.001*

Statistically significant results (<0.05) are indicated with an asterisk (*). AMP: Amphetamines. BNZ: Benzodiazepines. COC: Cocaine or metabolites. OPI: Opiates. THC: Cannabinoids. RT: Room temperature

DISCUSSION

In recent years, plastic sampling tubes have increasingly replaced glass tubes. Plastic tubes are inexpensive, break-resistant, safe for laboratory employees, and suitable for freezing samples (6). However, plastic tubes have been reported to cause adsorption or degradation of some analytes (14). Numerous studies have investigated the stability of therapeutic drugs in plastic or glass tubes (9,15); however, for drug abuse tests, there are only a few published reports (16,17,18). We compared the effects of glass versus plastic urine collection tubes on the results of five drug abuse tests and found no clinically significant differences and strong correlations between results in plastic and glass.

Previous studies reported that cannabinoids are hydrophobic molecules subject to adsorption to solid surfaces from aqueous solutions such as urine. Glass is reportedly an optimal material for the handling of cannabinoids (16,17). Bruno et al. evaluated the storage conditions of cannabinoids in plastic tubes versus glass tubes and found no significant difference in concentration between glass and plastic tubes at −20°C and −80°C. Furthermore, the study reported no degradation/adsorption in the first week of storage at +4°C (18). Our findings also

showed that glass or plastic tubes do not affect ~~the~~ cannabinoid levels, and we found no difference in the THC concentrations even after storage at room temperature for 4 hours in both plastic and glass tubes. The possible reason for findings differing from previous studies may be advances in the manufacturing of plastic tubes that reduce analyte adsorption or degradation of THC.

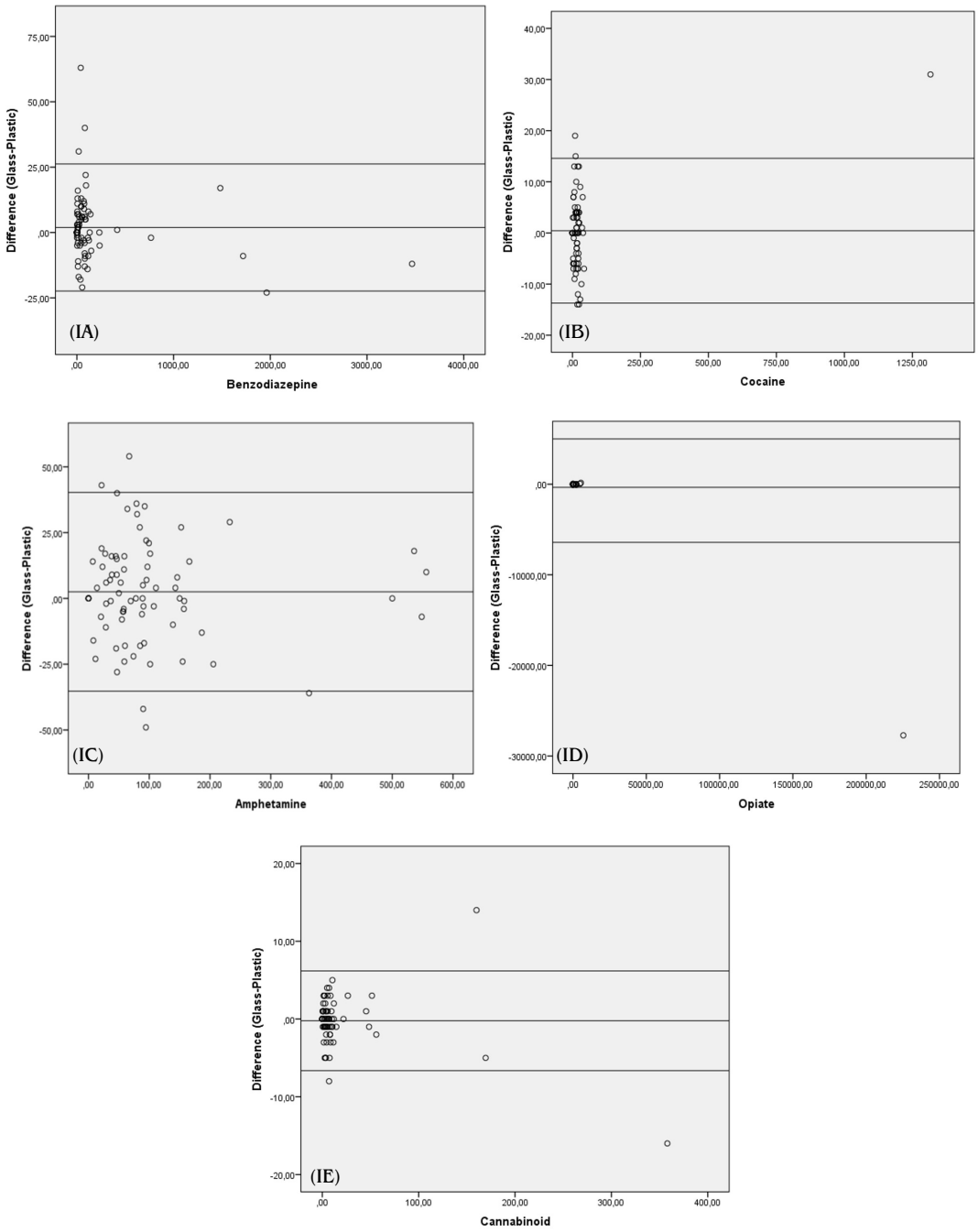

Figure 1. The Bland-Altman plots show the differences (y-axis, µg/L) and average values (x-axis, µg/L) for amphetamines (IA), benzodiazepines (IB), cocaine or metabolites (IC), opiates (ID), and cannabinoids (IE) between paired samples drawn into glass and plastic tubes

To our knowledge, this study is the first to compare the effects of glass versus plastic urine collection tubes on drug abuse tests of AMP, COC, BNZ, and OPI. Our findings showed no differences between glass and plastic tubes for AMP, BNZ, and COC levels. A statistically significant difference was found for OPI (Table 1), but this difference did not change the sample's positivity or negativity results based on the cut-off value. There were isolated statistically significant differences between glass and plastic tubes after 4 hours of storage for BNZ (Table 2), were an increase in BNZ concentration was observed. However, this difference was clinically insignificant. Although there were numerical differences in some drug results, it is important to emphasize that all measurements were compatible with each other based on the cut-off values. Literature indicates that all immunoassays do not perform equally well for some drugs (19). While quantitative methods may provide more accurate results, these methods require greater expertise, have longer processing times, and are costly (20). Regarding the statistically significant difference observed for opiates, we recommend that laboratories consider further validation studies, particularly using more precise analytical techniques such as GC-MS (Gas Chromatography-Mass Spectrometry), to ensure that differences between collection tube materials do not influence clinical decisions.

A possible limitation of our study is that most of the test results were below the cut-off value. Further studies with more abnormal samples should be conducted. Among the abnormal results in this study, there were no significant differences between glass and plastic collection tubes that changed the clinical decision. Additionally, as we conducted the study in affiliation with AMATEM, we faced difficulties in obtaining positive samples, which limited the robustness of our analysis.

CONCLUSION

Our study demonstrated no clinically significant differences between plastic and glass urine collection tubes for drug abuse tests, including AMP, COC, BNZ, OPI, and THC, even after short-term storage. These findings suggest that plastic tubes are a suitable alternative to glass tubes for drug abuse testing, though further studies with larger sample sizes and more precise testing methods, such as GC-MS are recommended to confirm these results.

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Araştırma Makalesi / *Research Article*

Is Capillary Zone Electrophoresis a Reliable Alternative for BCG-Based Albumin Measurement

Kapiller Zon Elektroforezi, BCG Yöntemiyle Albümin Ölçümünde Güvenilir Bir Alternatif mi?

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ABSTRACT

Objective: This study aimed to evaluate the agreement between albumin measurements obtained via capillary zone electrophoresis (CZE) and bromocresol green (BCG) methods and to determine their interchangeability in clinical practice. Additionally, it explored how these results vary across different protein patterns.

Materials and Methods: Albumin concentrations in 373 patients were measured using the CZE and BCG methods. Subgroup analyses were conducted based on albumin, total protein, IgG, gamma band percentages, and M protein concentrations. Agreement was assessed using Bland-Altman analysis, while constant, proportional, and random errors were analyzed with Passing-Bablok regression. Clinical significance was determined by comparing total allowable error (TEa) with total analytical error (TAE).

Results: CZE method yielded albumin levels that were, on average, 0.28 g/dL lower than those measured by BCG. Greater bias (12.5%) was observed at low albumin levels. Regression analysis revealed constant, proportional, and random errors. Subgroup analyses indicated that TAE exceeded the TEa threshold.

Conclusion: Significant analytical errors exist between albumin concentrations measured by CZE and BCG, making these methods non-interchangeable. Detailed subgroup analyses revealed that this discrepancy consistently persisted across different protein patterns. These findings emphasize the importance of cautious use of the CZE method in clinical decision-making.

Keywords: Albumin, Capillary Electrophoreses, Bromcresol Green, Reproducibility of Results, Bias

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ÖZET

Amaç: Bu çalışmanın amacı, kapiller zon elektroforezi (CZE) yöntemi ile bromokrezol yeşili (BCG) yöntemine dayalı albümin ölçümlerinin uyumunu değerlendirmek ve bu yöntemlerin klinik uygulamalarda birbirinin yerine kullanılıp kullanılamayacağını belirlemektir. Çalışma ayrıca, albümin ölçüm sonuçlarının farklı protein paternlerine göre nasıl değişkenlik gösterdiğini incelemiştir.

Gereç ve Yöntem: CZE ve BCG yöntemleriyle albümin konsantrasyonları 373 hastada ölçüldü. Albümin ve total protein seviyeleri, IgG konsantrasyonları, gama bandı yüzdesi ve M protein konsantrasyonları analiz edilerek alt gruplar oluşturuldu. İki yöntem arasındaki uyum Bland-Altman analizi ile değerlendirildi. Sabit, oransal ve rastgele hatalar Passing-Bablok regresyonu ile analiz edildi. Klinik anlamlılık, toplam kabul edilebilir hata (TEa) ile toplam analitik hata (TAE) değerlerinin karşılaştırılmasıyla değerlendirildi.

Bulgular: CZE yöntemi, albümin düzeylerini BCG yöntemine kıyasla ortalama 0.28 g/dL daha düşük ölçtü. Düşük albümin seviyelerinde (%12.5) daha yüksek yanlılık saptandı. Sabit, orantılı ve rastgele hataların mevcut olduğu görüldü. Alt grup analizleri, TAE'nin TEa'yı aştığını ortaya koydu.

Sonuç: CZE ve BCG yöntemleri arasında belirgin analitik hatalar saptanmış ve bu yöntemlerin birbirinin yerine kullanımı önerilmemiştir. Farklı protein paternlerinde yapılan detaylı alt grup analizleri, bu uyumsuzluğun tutarlı bir şekilde devam ettiğini göstermiştir. Bulgular, CZE yönteminin klinik karar süreçlerinde dikkatle değerlendirilmesi gerektiğini açıkça vurgulamaktadır.

Anahtar kelimeler: Albümin, Kapiller Elektroforez, Bromkrezol Yeşili Sonuçların tekrarlanabilirliği Yanlılık

INTRODUCTION

Human serum albumin (HSA) measurement serves various clinical purposes, including the assessment of nutritional status, liver and kidney function, coronary heart disease, multiple myeloma, neurometabolic disorders, diabetes (1), and the prediction of corrected calcium levels (2). Additionally, low albumin levels have been associated with increased mortality, while appropriate treatment has been shown to improve patient outcomes (3). Traditional HSA detection methods primarily rely on dye-binding and immunoassay techniques, which are widely used in clinical diagnostics (4). Over time, advanced technologies such as fluorescent probe assays, nano-material-based detection, and biosensors have been developed. However, despite these advancements, comprehensive reviews covering these methods remain scarce (5).

Capillary zone electrophoresis (CZE) is a widely used technique for detecting and monitoring monoclonal gammopathy. This method enables the electrophoretic separation of serum proteins, including albumin, by determining the percentages of protein fractions. It requires total serum protein concentration, measured by a different method, to convert these percentages into concentrations (6). Despite its widespread application, limited data exist regarding the agreement between HSA measurements obtained through CZE and other methods in medical laboratories (6,7).

This study aimed to evaluate the agreement between serum albumin concentrations obtained using capillary zone electrophoresis (CZE) and the bromocresol green (BCG) method, and to determine whether CZE can serve as a reliable alternative in clinical practice. Additionally, this study analyzed how discrepancies between these methods vary across different total protein concentrations and protein patterns, such as gamma band percentages, M protein concentrations, and IgG levels, through detailed subgroup analyses.

MATERIAL AND METHOD

Study Design: This cross-sectional, retrospective study included data from 373 patients aged 18–90 years, who had serum protein electrophoresis, total protein, albumin, and immunoglobulin results under the same barcode. Analyses were conducted in our laboratory over one year, from September 1, 2023, to September 1, 2024.

Patients with a hemolysis index >500, icterus index >20, lipemia index >550, CRP >5 mg/L, or biclonal gammopathy were excluded. Ethical approval was obtained from the local ethics committee (Decision number 22, dated November 28, 2023).

Categorization and Creation of Subgroups: Serum albumin concentrations measured by the bromocresol green (BCG) method and capillary zone electrophoresis (CZE) were first compared. Patients were then categorized into two groups based on albumin concentrations obtained using the direct measurement method:

- Albumin (g/dL): <3.5 and 3.5–5.2 (2 groups).

For a more detailed analysis, 11 additional subgroups were created based on laboratory reference ranges, categorized as follows:

- Total Protein (g/dL): <6.4 and 6.4–8.3 (2 groups)
- IgG (g/L): <7, 7–16, and >16 (3 groups)
- Gamma Band (%): <9.69, 9.69–18.9, and >18.9 (3 groups)
- M-Spike (g/dL): Absent, <0.1, and ≥0.1 (3 groups)

The M-spike was categorized using a threshold of 0.1 g/dL. This threshold ensures reliable quantification, as M-bands below this level, particularly in the presence of a polyclonal gammaglobulin background, are often reported as "trace" or "small band," reflecting their limited clinical significance. This categorization was selected to enhance measurement reliability and support clinical decision-making (8).

Capillary Zone Electrophoresis (CZE): The CZE analysis was performed using the Helena V8 Nexus instrument (Helena Biosciences, Gateshead, UK), equipped with eight fused silica capillary tubes (50 μm in diameter, 300 mm long). Migration occurred in a buffer with a pH of 9.9 under high voltage, while the temperature was maintained between 20 and 25 °C using a Peltier cooling system. The optical system included a deuterium lamp and a monochromator set to 214 nm. CZE determines the percentages of albumin, M protein, and other protein fractions converted to concentration equivalents using the total protein concentration.

Serum Total Protein, Albumin, and Immunoglobulin Measurements: Total protein, albumin, and immunoglobulin levels were analyzed on a Cobas c702 (Roche Diagnostics, Mannheim, Germany) autoanalyzer using the Biuret, bromocresol green (BCG), and immunoturbidimetric methods, respectively.

Statistical Analysis The comparison between the two methods was evaluated using a Bland-Altman plot to assess agreement and a Passing-Bablok regression analysis to determine the correlation. CUSUM test was used to validate the linearity of the Passing-Bablok regression analysis. According to interpretation guidelines, the methods are considered comparable within the studied concentration range if the 95% confidence interval (CI) of the intercept (a), representing the constant error, includes 0, and the CI of the slope (b), indicating proportional error, includes 1. A CI for the intercept excluding 0 indicates a constant error, while a CI for the slope excluding 1 suggests a proportional error (9).

Clinical significance was determined by evaluating whether the total analytical error (TAE) exceeded the total allowable error (TEa). TEa was calculated using intra-individual and inter-individual biological variations derived from the median albumin concentrations measured by the direct bromocresol green (BCG) method. TAE was calculated as the sum of constant error (a), proportional error (b−1), and random error (1.96×RSD [Residual Standard Deviation]). The factor 1.96 was used to account for the 95% confidence interval of the residual standard deviation, ensuring that random differences were evaluated within this statistical range. These analyses were

performed for all patients and across the 11 subgroups. Statistical analyses were conducted using MedCalc V22.023.

RESULTS

Albumin concentration data from the 373 patients included in the study are presented in Figures 1a and 1b. Figure 1a illustrates the Bland-Altman difference plot, comparing albumin concentrations obtained by the direct measurement method and capillary zone electrophoresis (CZE), with a mean bias of 0.28 g/dL (95% limits of agreement: -0.05 to 0.61). Figure 1b depicts the Passing-Bablok regression analysis, which assesses the correlation between the two methods using the same dataset. Both constant and proportional errors were observed in albumin measurements by CZE across the entire study group, with a correlation coefficient of $r^2 = 0.929$. The intercept of -0.6081 (95% CI: -0.7600 to -0.4554), which does not include zero, indicates a constant error, while the slope of 1.0774 (95% CI: 1.0417 to 1.1134), significantly deviating from 1, suggests a proportional error. Additionally, residual analysis revealed significant random error. As shown in the residuals plot (Figure 3), the residuals did not predominantly distribute within the expected range of ±1.96 × residual standard deviation (RSD: 0.1154; range:-0.2261 to 0.2261). Instead, the majority of the residuals were concentrated below zero, indicating a skewed distribution rather than randomness. This observation highlights the presence of substantial random error, further limiting the agreement between the two methods.

In addition, patients were grouped based on albumin concentrations obtained from the direct measurement method: those with concentrations <3.5 g/dL (n=27) and those with concentrations between 3.5–5.2 g/dL (n=344). Agreement analysis between direct measurement and CZE-derived albumin concentrations showed that for low albumin concentrations (<3.5 g/dL), the mean bias was 12.5% (95% limits of agreement: -11% to 36%), while for albumin concentrations within the reference range (3.5–5.2 g/dL), the mean bias decreased to 6.6% (95% limits of agreement: -1.7% to 14.9%). Only two patients had albumin concentrations >5.2 g/dL. These findings are illustrated in Figures 2a and 2b.

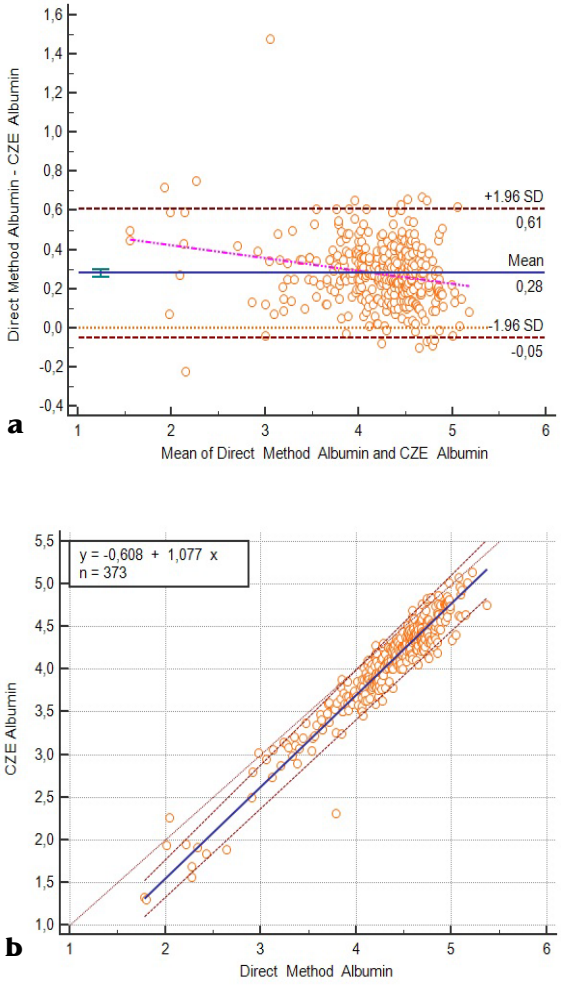

Figure 1. a. Bland-Altman plot comparing albumin concentrations obtained from the direct measurement method with those obtained from capillary zone electrophoresis (CZE) based on data from 373 patients. **b.** Passing-Bablok regression analysis comparing albumin concentrations obtained from the direct measurement method with those obtained from capillary zone electrophoresis (CZE) based on data from 373 patients.

The agreement and correlation between albumin concentrations measured by the direct method and CZE were analyzed across all patients and within 11 subgroups.

The Passing - Bablok regression results demonstrated constant error and proportional error in specific subgroups, while the Bland-Altman plot revealed varying mean bias values across the groups (Table 1). Bold values in Table 1 from the Passing-Bablok regression analysis indicate subgroups where no constant error or proportional error was observed, reflecting better agreement between the two measurement methods.

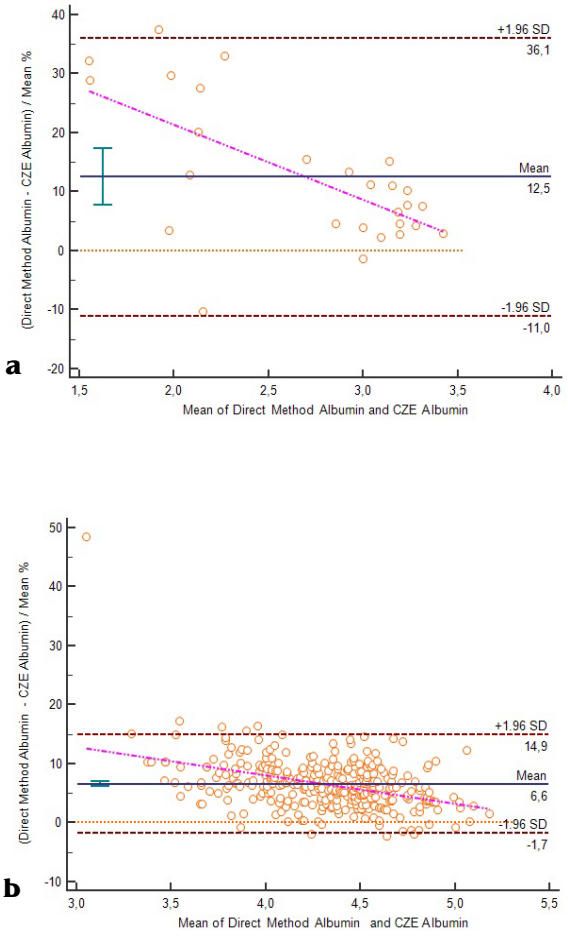

Figure 2. a. Bland-Altman plot comparing albumin concentrations obtained from the direct measurement method (BCG) with those obtained from capillary zone electrophoresis (CZE) for patients with albumin concentrations <3.5 g/dL (n=27). **b.** Bland-Altman plot comparing albumin concentrations obtained from the direct measurement method (BCG) with those obtained from capillary zone electrophoresis (CZE) for patients with 3.5-5.2 g/dL albumin concentrations (n= 346).

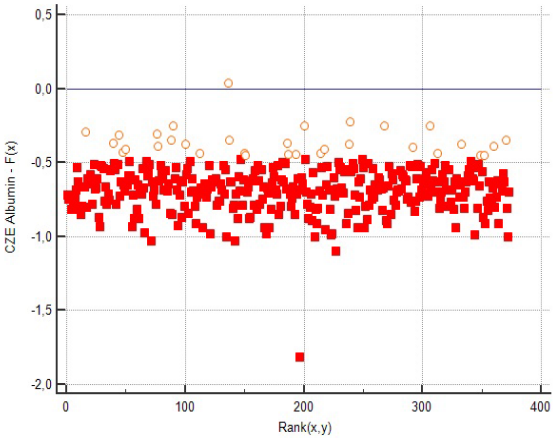

Figure 3. Residuals plot for albumin concentrations obtained using the CZE and BCG methods across the entire cohort (n=373).

Table 2 compares TEa and TAE for albumin concentrations obtained through direct measurement and CZE across the entire group and subgroups. The TAE was compared with the TEa, and these analyses were conducted separately for all patients as well as for each of the 11 subgroups created. TEa was calculated based on the Desirable Biological Variation Database specifications on Westgard's website, updated in 2014. The desirable TEa (%) was calculated as $<1.65(0.50 \times CVw) + 0.25 \sqrt{(CV^2w + CV^2g)}$, where CVw represents within-subject biological variation, CVg represents between-subject biological variation, and TEa denotes the desirable specification for allowable total error. Accordingly, albumin's desirable TEa was established at 4.07% (10-12). Total allowable errors for each group were calculated in g/dL using the median albumin concentrations obtained from the entire group and each of the subgroups. These values were then compared with the TAE obtained from the Passing-Bablok regression analysis as described in the statistical analysis section. As shown in Table 2, the TAE values in the overall group and the subgroups were significantly higher than the calculated TEa values.

Table 1. Passing-Bablok Regression and Bland-Altman Plot Analysis of Albumin Concentrations: Direct Measurement Method vs. CZE Across All Patients and Subgroups.
Tablo 1. Albümin Konsantrasyonlarının Passing-Bablok Regresyon ve Bland-Altman Grafiği Analizi: Tüm Hastalar ve Alt Gruplar İçin Doğrudan Ölçüm Yöntemi ile CZE'nin Karşılaştırılması.

Groups		Passing-Bablok Regression Analysis				Bland-Altman Plot Analysis	
	n	Regression Equation	Intercept, 95% CI	Slope, 95% CI	Cusum Test	Mean Bias (g/dL)	p
All group	373	y = -0,612225 + 1,078331 x	-0,7647 to -0,4600	1,0427 to 1,1145	(P=0,49)	0,28	<0,0001
Albumin (<3.5)	27	y = -0,903180 + 1,210693 x	-1,5154 to -0,3413	1,0265 to 1,4079	(P=1,00)	0,29	<0,0001
Albumin (3.5-5.2)	346	y = -0,748478 + 1,108696 x	-0,9565 to -0,5389	1,0606 to 1,1558	(P=0,61)	0,28	<0,0001
TP (<6.4)	83	y = -0,423067 + 1,025410 x	-0,6901 to -0,1463	**0,9550 to 1,0933**	(P=0,40)	0,33	<0,0001
TP (6.4-8.3)	290	y = -0,627514 + 1,083834 x	-0,8568 to -0,4057	1,0327 to 1,1354	(P=0,33)	0,26	<0,0001
IgG (<7)	76	y = -0,492885 + 1,041147 x	-0,7509 to -0,2217	**0,9795 to 1,1030**	(P=0,71)	0,33	<0,0001
IgG (7-16)	251	y = -0,735088 + 1,106536 x	-0,9606 to -0,5113	1,0542 to 1,1573	(P=0,50)	0,28	<0,0001
IgG (>16)	46	y = -0,409529 + 1,043742 x	**-0,7516 to 0,04566**	**0,9442 to 1,1249**	(P=0,90)	0,20	<0,0001
Gamma (<9,69)	84	y = -0,594329 + 1,065281 x	-0,8916 to -0,2873	**0,9941 to 1,1340**	(P=0,92)	0,33	<0,0001
Gamma (9,69-18,9)	225	y = -0,754002 + 1,112379 x	-1,0155 to -0,5348	1,0621 to 1,1684	(P=0,53)	0,27	<0,0001
Gamma (>18,9)	64	y = -0,491639 + 1,063763 x	-0,8515 to -0,1050	**0,9752 to 1,1540**	(P=0,81)	0,23	<0,0001
M-Spike (Absent)	276	y = -0,665087 + 1,093554 x	-0,8309 to -0,4956	1,0542 to 1,1309	(P=0,30)	0,27	<0,0001
M-Spike (<0,1)	10	y = -1,248418 + 1,211516 x	**-3,4031 to 0,2171**	**0,8812 to 1,7061**	(P=0,77)	0,31	<0,0001
M-Spike (≥ 0,1)	87	y = -0,379982 + 1,016580 x	**-0,7836 to 0,01915**	**0,9261 to 1,1087**	(P=0,43)	0,32	<0,0001

CZE: Capillary zone electrophoresis, TP: Total protein, Gamma: The percentage of the gamma region in CZE, M-spike: M protein detected by CZE

Table 2. Comparison of Total Allowable Error (TEa) and Total Analytical Error (TAE) with its Components for Albumin Concentrations: Direct Measurement vs CZE
Tablo 2. Albümin Konsantrasyonları İçin Toplam İzin Verilebilir Hata (TEa) ve Toplam Analitik Hata (TAE) Komponentlerinin Karşılaştırılması: Doğrudan Ölçüm Yöntemi ve CZE.

Groups	n	Median g/dL	TEa (g/dL)	CE	RE	PE	TAE
All group	373	4.46	0.18	0.61	0.22	0.07	**0.90**
Albumin (<3.5)	27	3.06	0.13	0.90	0.29	0.21	**1.40**
Albumin (3.5-5.2)	346	4.49	0.18	0.75	0.22	0.11	**1.08**
TP (<6.4)	83	4.05	0.16	0.42	0.24	0.03	**0.69**
TP (6.4-8.3)	290	4.56	0.19	0.63	0.22	0.08	**0.93**
IgG (<7)	76	4.41	0.18	0.49	0.21	0.04	**0.74**
IgG (7-16)	251	4.49	0.18	0.74	0.24	0.11	**1.09**
IgG (>16)	46	4.34	0.18	0.41	0.18	0.04	**0.63**
Gamma (<9.69)	84	4.46	0.18	0.59	0.21	0.07	**0.87**
Gamma (9.69-18.9)	225	4.53	0.18	0.75	0.23	0.11	**1.09**
Gamma (>18.9)	64	4.19	0.17	0.49	0.22	0.06	**0.77**
M-Spike (Absent)	276	4.47	0.18	0.67	0.21	0.09	**0.97**
M-Spike (<0.1)	10	4.45	0.18	1.25	0.16	0.21	**1.62**
M-Spike (≥ 0.1)	87	4.27	0.17	0.38	0.28	0.02	**0.68**

CE: Constant Error (Intercept in Passing-Bablok regression). **CZE**: Capillary Zone Electrophoresis (A method for serum protein separation). **Gamma**: Gamma Region Percentage in CZE. **M-spike:** M Protein detected by CZE. **PE:** Proportional Error (Deviation of slope "b" from 1). **RE:** Random Error (1.96 × Residual Standard Deviation). **TAE:** Total Analytical Error (Sum of CE, PE, and RE). **TEa:** Total Error Allowable (Calculated based on biological variations; set at 4.07%, determined using the median albumin values for the overall cohort and subgroups). **TP:** Total Protein

DISCUSSION

Numerous analytical methods are available for determining human serum albumin (HSA) concentration, ranging from traditional dye-binding and immunological methods to advanced techniques such as fluorescent probes, nanoparticles, biosensors, chromatographic techniques, and electrophoresis-based methods. Despite these advancements, dye-binding and immunological approaches remain the most commonly used in routine clinical practice due to their simplicity and cost-effectiveness. However, the clinical implementation of newer techniques, such as fluorescent probes and biosensors, requires further validation and optimization (5).

In our study of 373 patients, we observed that albumin concentrations measured by the CZE method were consistently lower compared to the direct BCG method. This underestimation was particularly pronounced at low albumin levels (<3.5 g/dL), with a discrepancy of 12.5%, while within the reference range (3.5–5.2 g/dL), the bias decreased to 6.6%.

Statistically significant constant, proportional, and random errors were observed in albumin measurements obtained via the CZE and BCG methods across the entire cohort and all 11 subgroups. Constant and proportional errors were demonstrated by the intercept and slope of the Passing-Bablok regression analysis, while residual analysis revealed significant random error. Most residuals did not lie within the expected range of ±1.96 times the residual standard deviation (RSD: 0.1154; interval: -0.2261 to 0.2261) and were predominantly distributed below zero. Furthermore, the TAE values in all groups exceeded the predefined TEa limits,

emphasizing that albumin concentrations measured by the CZE method may have clinical implications and potentially affect patient management.

In certain subgroups, the confidence intervals (CIs) of the intercept and slope were wider, suggesting potential challenges in interpreting the agreement between the methods. Notably, in cases where the CI of the intercept includes 0 and the CI of the slope includes 1, the methods might appear to be in agreement. However, as highlighted by Mayer et al. (13), this perceived agreement could be an artifact of small sample sizes, which tend to produce larger CIs. This methodological limitation is further supported by Ludbrook (14), who emphasized that method comparison studies with small sample sizes are inherently biased toward concluding agreement between laboratory methods. For robust Passing-Bablok regression analysis, a sample size of at least 50 is recommended, as stated by Passing and Bablok (15). Therefore, the apparent agreement observed in certain subgroups with limited sample sizes should be interpreted cautiously, as it may not reflect true methodological compatibility.

Previous studies have demonstrated that dye-binding methods based on bromocresol green (BCG) interact not only with albumin but also with other acute-phase proteins, such as α-globulins, particularly in samples with low albumin concentrations (<2.5–3.5 g/dL) (16). This interaction leads to a positive bias in albumin measurement, with discrepancies of up to 1.0 g/dL compared to bromocresol purple (BCP) or nephelometric (IN) methods (17). The positive bias observed with BCG is primarily attributed to its reactivity with proteins such as α2-macroglobulins and haptoglobin (18). Consequently, BCG-based assays may yield inconsistent results and should be interpreted cautiously, particularly in clinical scenarios such as nephrotic syndrome, where α2-macroglobulin levels are significantly elevated (19). Understanding these limitations is essential for ensuring the accurate application of the BCG method in our laboratory and for the reliable interpretation of clinical results.

Our study demonstrated that the CZE method tends to underestimate albumin levels compared to the BCG method, particularly in subgroups with and without M-spikes. However, Padelli et al. (7) reported a systematic overestimation of albumin levels by the CZE method in monoclonal gammopathy patients. This discrepancy may be due to methodological differences and the varying impact of monoclonal immunoglobulins on albumin measurements. Padelli et al. also observed a proportional overestimation correlated with increasing monoclonal immunoglobulin concentrations, which highlights the variability in CZE performance across different clinical scenarios.

The determination of albumin levels using the CZE method heavily relies on the accuracy of serum total protein measurements, which are performed as a separate test (6,7). In our laboratory, serum total protein concentrations are measured using the Biuret method, which, according to the kit insert, is prone to interference from conditions such as hemolysis, lipemia, icterus, and high dextran concentrations. However, as patients with values exceeding these thresholds were excluded from our study, we do not believe that these interferences affected the results. Additionally, rare cases of gammopathies, especially those involving IgM-type paraproteins (e.g., Waldenström's macroglobulinemia), can lead to unreliable total protein results measured by the Biuret method (20).

Previous studies have reported a lack of agreement between the CZE and BCG methods. Our study is among the first to systematically investigate how this disagreement varies across different protein patterns, including gamma band percentages, M protein concentrations, and IgG levels. These findings emphasize the

limitations of substituting one method for the other and underscore the impact of protein patterns on method agreement in clinical applications. Additionally, our results suggest that discrepancies observed in subgroups with absent and varying levels of M-spikes cannot be solely attributed to interference from Biuret-based total protein measurements. This indicates that other factors, beyond total protein interference, contribute to the observed lack of agreement between the methods.

One of the limitations of this study is the limited number of patients with low albumin concentrations (<3.5 g/dL). Although the study included 373 patients, only 27 patients fell within this range, which may limit the generalizability of the findings for low albumin levels. Additionally, the observed residuals demonstrated a skewed distribution, predominantly below zero, indicating significant random error that could influence the agreement between the methods. These findings suggest that further studies with larger sample sizes, particularly in the low albumin concentration range, are necessary to validate the results and explore the impact of random error more comprehensively. Moreover, the influence of other potential factors, such as variations in total protein measurements and their methodological interferences, should be considered in future research.

CONCLUSION

This study demonstrated that albumin concentrations measured using the CZE and BCG methods are not interchangeable due to statistically and clinically significant constant, proportional, and random errors. Careful selection of the albumin measurement method is crucial, particularly in clinical scenarios such as kidney and liver diseases or nutritional assessments, where accurate albumin values are critical for patient management and therapeutic decision-making.

Moreover, while previous studies have reported a lack of agreement between these methods, our study uniquely investigated how this disagreement varies across different protein patterns, such as gamma band percentages, M protein concentrations, and IgG levels, through detailed subgroup analyses. These findings underscore the limitations of the CZE method and emphasize the need for careful consideration when interpreting its results in clinical practice.

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Derleme Makalesi / *Review Article*

Hemofilinin Tanı ve İzleminde Klinik Laboratuvarların Rolü

The Role of Clinical Laboratories in the Diagnosis and Monitoring of Hemophilia

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ÖZET

Hemofili A ve B, sırasıyla faktör VIII ve faktör IX genlerindeki mutasyonların neden olduğu pıhtılaşmanın yokluğu veya azalmış aktivitesi ile karakterize X'e bağlı kanama bozukluklarıdır. Hemofili tanısı, hemofilinin klinik özelliklerini ve semptomlarını anlama, kanamanın potansiyel nedenini belirlemeye yönelik testler olan protrombin zamanı, aktive kısmi tromboplastin zamanı, trombosit sayısı ve fonksiyonu gibi tarama testlerinin kullanılması ve yorumlanması ile faktör analizleri ve uygun yöntemlerle tanının doğrulanması gibi spesifik araştırmalara dayanır. Bu derlemede Hemofili tanı ve takibinde kullanılan biyokimyasal testler, kullanım algoritmaları ile karışım testleri, faktör ve faktör inhibitör testleri hakkında ayrıntılı bilgi verilmesi amaçlanmıştır.

Anahtar Sözcükler: Hemofili, Bedhesta, Faktör, İnhibitör

ABSTRACT

Hemophilia A and B are X-linked bleeding disorders characterized by the absence or reduced activity of coagulation caused by mutations in the factor VIII and factor IX genes, respectively. Diagnosis of hemophilia is based on understanding the clinical features and symptoms of hemophilia, screening tests to determine the potential cause of bleeding such as prothrombin time, activated partial thromboplastin time, platelet count and function, and specific investigations such as factor analysis and confirmation of the diagnosis with appropriate methods. In this review, it is aimed to give detailed information about the biochemical tests used in the diagnosis and follow-up of Hemophilia, their usage algorithms, mixture tests, factor and factor inhibitor tests.

Keywords: Hemophilia, Bedhesta, Factor, Inhibitor

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GİRİŞ

Hemofili, hemofili A ve B olarak adlandırılan pıhtılaşma faktörleri VIII (FVIII) ve IX (FIX)'un eksikliği ile karakterize edilen, X'e bağlı konjenital bir kanama bozukluğudur. Hemofili hastalarında yaralanma, diş çekimi ve cerrahi işlemler sonrasında uzun süreli kanama görülür (1,2). FVIII ve FIX, intrensek yolda yer alan pıhtılaşma faktörleridir. Sonuç olarak, pıhtılaşma faktörlerindeki eksiklik, aktive kısmi tromboplastin zamanı (aPTZ) uzaması ile sonuçlanır (2,3). Laboratuvar bulguları tipik olarak normal protrombin zamanı (PZ), normal trombin zamanı, normal trombosit sayısı ve uzamış aPTZ'yi gösterir. Kanama ile başvuran hastalardaki tarama testleri Tablo 1'de gösterilmiştir.

Hemofili'de kanama ataklarının tanısı ve sıklığı eksik faktör aktivitesi ile ilişkilidir. Şiddetli hemofilide faktör aktivitesi düzeyleri 1 IU/dL'nin (%1) altında, orta hemofilide 1-5 IU/dL (%1-5) arasında, hafif hemofilide ise 5-40 IU/dL (%5-40) arasında saptanır (1,2). Şiddetli hemofili A'lı bireyler, genellikle yaşamın ilk iki yılında kanama veya bilinen bir aile hemofili öyküsü nedeniyle teşhis edilir. Profilaktik tedavi almayan bireylerde her ay ortalama iki ile beş arasında spontan kanama atağı görülebilir. Orta derecede hemofili A hastalarında, kişiden kişiye değişmekle birlikte nadiren spontan kanama görülür, küçük bir travmadan sonra uzamış veya gecikmiş kanamaları olur ve genellikle beş ile altı yaşından önce teşhis edilirler. Kanama ataklarının sıklığı genellikle ayda bir ile yılda bir arasında değişir. Hafif hemofili A hastalarında ise spontan kanama atakları görülmez ancak ameliyat öncesi ve sonrası tedavi uygulanmadığında anormal kanama meydana gelebilir. Kanama ataklarının sıklığı tipik olarak yılda bir ile on yılda bir arasında değişir. Hafif hemofili A'lı bireylere genellikle yaşamın ilerleyen zamanlarına kadar teşhis konulamaz (1).

Edinsel hemofili (EH) ise FVIII aktivitesini inhibe eden otoantikorların varlığı ile karakterize nadir bir hastalıktır. Ancak FV, VII, IX, X, XII ve XIII gibi diğer pıhtılaşma faktörlerini içeren vakalar da rapor edilmiştir. Yeni başlayan kanama ve izole aPTZ uzaması ile başvuran hastalarda EH tanısı düşünülmelidir. Edinsel hemofili A (EHA) tanısı, FVIII aktivitesinin azalmasına ve inhibitör olarak da adlandırılan nötralize edici anti-faktör VIII antikorlarının varlığına dayanır. EHA olgularının yaklaşık %50'sinde neden bilinmemektedir, geri kalan olgular ise sıklıkla altta yatan maligniteler, enfeksiyonlar, ilaç tedavileri veya romatoid artrit gibi otoimmün hastalıklarla ilişkilidir (1,4,5).

aPTZ, intrensek ve ortak pıhtılaşma yollarının aktivitesini değerlendirmek için kullanılan pıhtı bazlı bir testtir. aPTZ kitinde intrensek aktivitenin ölçülebilmesi amacıyla prokoagulan fosfolipid (doku faktörü içermeyen kısmi tromboplastin) ve aktivatör bulunur. Fosfolipidler, pıhtılaşma faktörlerinin etkileşimi için uygun bir yüzey ortamı sağlar (6). İnorganik veya organik aktivatörler (örneğin kaolin, mikronize silika, ellagik asit veya bitkisel fosfatidler) ile temas faktörlerinin (örneğin yüksek molekül ağırlıklı kininojen, prekallikrein ve faktör XII ve XI) aktivasyonu başlatılır. Uzamış aPTZ, muhtemelen faktör XII, XI, IX veya VIII'deki eksikliklere bağlı olarak intrensek yoldaki yetersizliği gösterir. Ek olarak, uzamış aPTZ, ortak yoldaki eksiklikleri (faktör V, X, II veya fibrinojen) ve spesifik inhibitörlerin veya heparin gibi antikoagülan ilaçların varlığını gösterebilir (Tablo 2) (3,7).

aPTZ reaktifleri, pıhtılaşma faktörü eksikliklerini tespit etmek, lupus antikoagülanlarını tahmin etmek ve heparin hassasiyetlerini değerlendirmek için değişen hassasiyetler sergiler. aPTZ kitlerindeki faktör hassasiyeti; kullanılan aPTZ kit reaktifi yanı sıra reaktif hassasiyetini belirlemede kullanılan faktör eksik plazma ve normal plazma havuzu (NPP)'na da bağlıdır. aPTZ reaktiflerinde bulunan fosfolipid ve yağ asidi bileşimi standardize değildir. Fosfolipidler, hayvan plasenta veya beyninden köken alabildiği gibi bitkisel kökenli de olabilmektedir. Bu nedenle aPTZ kitlerindeki faktör ve lupus antikoagulanı hassasiyeti farklılıklar gösterir (8). Çoğu aPTZ reaktifi, orta veya şiddetli hemofili hastalarında düşük sonuçlar üretse

de tüm reaktifler hafif hemofiliyi doğru şekilde tanımlama yeteneğine sahip değildir. Bu nedenle, hafiften şiddetliye kadar tüm hemofili türlerini tespit edebilen bir aPTZ reaktifinin seçilmesi, doğru tanı için çok önemlidir (7).

FAKTÖR ANALİZLERİ

Faktör analizleri hemofilinin ciddiyetini belirlemek, ameliyattan önce pıhtılaşma durumunu değerlendirmek ve profilaktik tedaviyi optimize etmek için gereklidir. Tedavi sırasında faktör aktivitesinde bir azalma, inhibitörlerin gelişimini gösterebilir. Faktör analizleri tek aşamalı, iki aşamalı veya kromojenik yöntemler kullanılarak yapılabilir (7).

Doğru faktör aktivitesi sonuçları elde etmek için numunelerin uygun şekilde alınması, santrifüj edilmesi ve saklanması çok önemlidir. Numuneler %3,2 trisodyum sitrat dihidrat'lı tüpe alınmalı ve 1500 g'de en az 15 dakika oda ısısında santrifüj edilmelidir. Örnekler, alım saatinden sonraki 4 saat içinde analiz edilmelidir. Santrifüj edilen veya tam kan halinde bulunan örneklerin 2-8 °C'de bekletilmesi önerilmez. Bekletildiği durumda FVII aktivasyonu ile FVIII ve von Willebrand faktör (vWf) aktivitesinde azalma görülür. Bu 4 saat süre içinde analiz edilemeyecek örnekler plazması ayrılarak -20°C'de 2 hafta veya -70°C'de 1 yıl dondurularak saklanmalıdır. Hemolizli örneklerden kaçınılmalıdır. Hemoliz durumunda açığa çıkan hücre içi maddeler ve fosfolipid gibi membran molekülleri faktör aktivasyonuna sebep olabilmektedir (9).

%3,2 trisodyum sitrat dihidrat'lı tüpe kan alımının hedef düzeye kıyasla en az %80 dolulukta olması gerekmektedir (2). İnflamasyon, stres, ağır egzersiz ve hamilelikte FVIII ve vWf düzeyleri artabileceği için tanı ve tedavi izleminde dikkatli olunmalıdır (10,11). Çalışmaya başlamadan önce dondurulmuş örnekler 4-5 dakika 37 °C su banyosunda bekletilerek hızlı bir şekilde çözülmelidir (2).

Tablo 1. Kanamada Tarama Testleri ve Yorumlanması
Table 1. Bleeding Screening Tests and Their Interpretations.

	PZ	aPTZ	Trombosit sayısı
Normal	Normal	Normal	Normal
Hemofili	Normal	Uzamış	Normal
vWH	Normal	Normal/ uzamış	Normal/ azalmış
Trombosit defekti	Normal	Normal	Normal/azalmış

PZ, protrombin zamanı; aPTZ, aktive kısmi tromboplastin zamanı; vWH, von Willebrand hastalığı

Tablo 2. İzole Uzamış aPTZ Sebepleri ve Eşlik Eden Bulgular
Table 2. Causes of Isolated Prolonged aPTT and Associated Findings.

İzole Uzamış aPTZ Sebepleri	Klinik/Eşlik Eden Bulgu
Hemofili A (FVIII eksikliği)	Kanama hikayesi var
Hemofili B (FIX eksikliği)	Kanama hikayesi var
F XI eksikliği	Kanama hikayesi var
F XII eksikliği	Kanama yok
Prekallikrein eksikliği	Kanama yok
Yüksek molekül ağırlıklı kininojen eksikliği	Kanama yok
Spesifik inhibitör (FVIII, IX, XI, XII)	Yeni başlayan kanama hikayesi
Lupus antikoagülanı varlığı	Tromboz
Antikoagulan kullanımı (Heparin, düşük molekül ağırlıklı Heparin ..)	İlaç kullanım hikayesi

aPTZ, aktive kısmi tromboplastin zamanı

FVIII aktivitesi azalmış tüm hastalarda von Willebrand hastalığı (vWh) dışlanmalıdır. Tip 2N vWh'da FVIII aktivitesi düşüklüğü yanında normal vWf antijen (vWf:Ag) düzeyi olması nedeniyle hastaların hafif hemofili A ile karıştırılmaması için ilk tanıda laboratuvar değerlendirilmesinin geniş ve tam olarak yapılması gereklidir (2).

1. Tek Aşamalı Yöntem (One Stage Assay)

Temel olarak aPTZ'ye dayanan tek aşamalı pıhtı bazlı test, klinik laboratuvarlarda en sık kullanılan yöntemdir. Tek aşamalı analizler, değerlendirilen spesifik faktöre bağlı olarak aPTZ veya PZ'ye dayanabilir. Bu testte, hasta plazmasının tampon çözelti ile dilüsyonları (1:10, 1:20, 1:40) hazırlanır. Her dilüsyon, faktör eksik plazma kit reaktifi ile 1:1 oranında karıştırılır ve ardından pıhtılaşma süreci başlatılır. Faktör eksik plazma kit reaktifinde yalnızca ölçülecek spesifik faktör bulunmaz ancak diğer tüm pıhtılaşma faktörleri yaklaşık 100 IU/dL kadar bulunur. Böylece sonuçlar sadece hasta plazmasındaki ölçülmek istenen faktörün düzeyine bağlı olur. Hastanın dilüsyonlu plazmalarından elde edilen sonuçların kalibrasyon eğrisi ile karşılaştırılmasıyla test sonucu elde edilir. En az 3 dilüsyon yapılması ve eğrinin kalibrasyon eğrisine paralel olması istenir. Eğer hasta sonuçlarındaki eğri kalibrasyon eğrisine paralel değil ise hasta örneğinde inhibitör olabileceği düşünülmeli ve ek dilüsyonlar ile eğri paralel hale getirilmeye çalışılmalıdır. Elde edilen sonuç dilüsyon faktörü ile çarpılarak hasta sonucu elde edilir (12).

Tek aşamalı aPTZ'ye dayanan faktör testlerinde ölçüm, lupus antikoagülanı, antikoagülan kullanımı ve spesifik faktör inhibitörlerin varlığı ile interferans gösterebilir. aPTZ temelli tek aşamalı analizler, intrensek faktörlerin aktivitesini değerlendirmek için kullanılırken, PZ temelli analizler öncelikle FVII'yi ve ortak pıhtılaşma yolunda yer alan faktörleri değerlendirmek için kullanılır (12).

Faktör eksik ticari plazmalar konjenital eksikliği bulunan hastalardan ya da sağlıklı hasta plazmalarının immunodeplesyonu veya kimyasal ön işlem ile elde edilebilir (8). Faktör FVIII eksik plazma kit reaktiflerinin <1 IU/dL FVIII içermesi ve diğer tüm faktörleri normal düzeyde içermesi zorunlu kılınmıştır. Faktör IX ölçümleri için de aynısı geçerlidir (2).

2. İki Aşamalı Yöntem (Two Stage Assay)

İki aşamalı analiz, faktör aktivitesini değerlendirmek için kullanılan diğer bir yöntemdir. İlk aşamada, FVIII ölçümü için hastanın plazmasına kalsiyum, fosfolipid, saflaştırılmış aktive FIX (FIXa) ve FX eklenerek aktive faktör X (FXa) oluşması sağlanır. İkinci aşamada ise, bu karışıma NPP eklenir ve ilk aşamada oluşan FXa'dan fibrin oluşturulur. Pıhtılaşma zamanı ilk reaksiyonda oluşan FXa miktarına bağlıdır. İki aşamalı test, heparin veya doğrudan trombin inhibitör ilaç kullanımından tek aşamalı teste göre daha az etkilenir. Ancak FXa inhibitörlerinin varlığı sonuçları etkileyebilir (12).

3. Kromojenik Yöntem

İki aşamalı ölçümle benzer prensipte çalışır. İlk aşamada, FVIII ölçümü için hastanın plazmasına kalsiyum, fosfolipid, saflaştırılmış FIXa ve FX eklenerek FXa oluşması sağlanır. İkinci aşamada ise FXa'dan enzimatik olarak salınan ve kromojenik bir substrat olan p-nitroaniline'nin absorbansı 405 nm'de ölçülür. İkinci aşamaya, kromojenik substratın azalmasını engellemek için trombin inhibitörü sıklıkla eklenir (13).

Kromojenik yöntem, tek aşamalı yöntemden sonra FVIII analizlerinde en sık kullanılan ikinci yöntemdir. FIX ölçümleri için de kromojenik yöntem kullanılmaya başlanmıştır (14-18). Kihlberg ve ark. kromojenik yöntemle ölçülen FIX sonuçlarının tek aşamalı yönteme göre klinik ile daha uyumlu olduğunu göstermişlerdir (17). Kromojenik FVIII analizleri genellikle tek aşamalı pıhtı temelli FVIII aktivite analizlerinden daha pahalıdır (19). İki aşamalı ve kromojenik yöntemin tek aşamalı yönteme göre lupus antikoagülanlarına daha az duyarlı olduğu bazı araştırmalarda gösterilmiştir (12).

Genetik olarak doğrulanmış bazı hafif hemofili A olgularında tanı için tek aşamalı test kullanıldığında FVIII aktivitesinin normal seviyede, ancak kromojenik veya iki aşamalı yöntem kullanıldığında ise azalmış düzeyde olduğu gösterilmiştir (20-24). Bazı çalışmalarda ise bu durumun tam tersi bulunmuştur. (Tablo 3) (25,26).

Dünya Hemofili Federasyonu (WFH), hemofili A'nın tüm hastalık tiplerinin saptanması için ilk tanıda tek aşamalı ve kromojenik yöntemin beraber kullanılması gerektiğini bildirmiştir. Testlerden biri normal FVIII aktivitesi gösterse dahi diğer yöntemde de ölçüm yapılmalıdır. WFH, hemofili B'nın ilk tanı aşamasında ise tek aşamalı yöntemin kullanılmasını önerir. Kromojenik yöntemin kullanılması için ise henüz yeterli çalışma bulunmadığı belirtilmiştir. Tek aşamalı veya kromojenik yöntem ile FVIII veya FIX çalışıldığında kalibrasyon için kullanılan referans materyal veya standart plazmaların Dünya Sağlık Örgütü (WHO) standartları tarafından izlenebilir olması gerekmektedir. Referans plazma olarak NPP kullanıldığında hasta sonuçları yüzde olarak verilebilmektedir. Ancak bu durum WHO standartları uygun olmaması sebebiyle önerilmez. WFH, FVIII ve FIX sonuçlarının IU/dL veya IU/mL olarak verilmesini tavsiye eder (2).

İNHİBİTÖRLER

Pıhtılaşma faktörü inhibitörleri faktör etkisini etkisiz hale getiren (Tip 1) ve etkisiz hale getirmeyen (Tip 2) olarak 2 tipte görülür. Etkisiz hale getiren antikorlar faktör inaktivasyonu ile sonuçlanır. Etkisiz hale getirmeyen antikorlar ise fonksiyonu olmayan epitopları hedef alırlar. Her iki tipte de alloantikor ve otoantikorlar bulunabilir. Alloantikorlar faktör aktivitesinde tam bir inhibisyon yaratabilir. Otoantikorlar ise EHA, hamilelik ve yaşlılık gibi durumlarda görülebilen sınırlı faktör inhibisyonuna sebep olan antikorladır (8).

Hemofilideki inhibitörler, infüze edilen ekzojen pıhtılaşma faktörü konsantrelerinine karşı gelişen FVIII veya FIX' u nötralize eden IgG tipinde alloantikorlardır. Pıhtılaşma faktörü konsantreleri replasman tedavisine yanıt veren ancak artık yanıt alınamayan her hemofili hastasında yeni bir inhibitörün varlığından şüphelenilmelidir (2)

Tablo 3. Faktör VIII Aktivitesinin Ölçümünde Tek Aşamalı Yöntem ile Kromojenik Yöntemin Karşılaştırılması
Table 3. Comparison of One-Stage Method and Chromogenic Method for Measuring Factor VIII Activity.

	Tek Aşamalı Yöntem	**Kromojenik Yöntem**
Avantajları	1. Basit, hızlı ve ucuz 2. Klinik izlem için en yaygın kullanılan yöntem	1.Ölçümde FVIII aktivasyonu olmaz 2. FVIII eksik plazma gerekli değildir 3. Tüm FVIII düzeylerinin ölçümü için uygundur 4.LA'ya karşı duyarsız 5.Laboratuvarlar arası değişkenlik daha az
Sınırlılıkları	1.Faktör aktivasyonuna duyarlı 2.FVIII eksik plazma gerekli 3.Yüksek Laboratuvarlar arası değişkenlik 4.LA, heparin, direkt oral antikoagülan ilaçlarına duyarlı 5.Bazı hafif hemofili A fenotiplerinde FVIII aktivitesi yüksek ölçülebilir	1.Tek aşamalı yöntem'den daha pahalı 2.Tek aşamalı yöntem kadar yaygın kullanılmaz 3.Teknik olarak daha karışık 4.Direkt oral antikoagülan ilaçlarına duyarlı 5.Bazı hafif hemofili A fenotiplerinde FVIII aktivitesi yüksek ölçülebilir

FVIII, Faktör VIII; LA, Lupus Antikoagulanı

Orta veya hafif hemofili hastalarına göre şiddetli hemofili hastalarında ve hemofili B hastalarına kıyasla hemofili A hastalarında inhibitörlere daha sık rastlanır. Siyah ve Hispanik etnik kökene sahip hemofili hastalarında inhibitörün prevalansı daha yüksek gözlenmektedir. Şiddetli hemofili A hastalarında, yirmi adet FVIII replasman tedavisi sonrası %30 oranda FVIII'e karşı alloimmün inhibitörler gelişir (1,4).

İnhibitörlü hemofili hastalarında, inhibitörü olmayanlara göre kanamanın kontrol edilmesi daha büyük bir zorluktur. Bunun yanında inhibitörü olan hemofili hastalarında kas-iskelet sistemi komplikasyonları, ağrı ve yaşam kalitesinin düşmesi gibi sorunlar da görülmektedir. (2).

Lupus antikoagülanı (LA) gibi nonspesifik inhibitörler in vitro fosfolipid bağımlı faktör testlerinde interferansa sebep olabilir. Bu antikorlar protein ve fosfolipid komplekslerine karşı oluşur ve pıhtılaşma faktörlerini inhibe etmezler. Antikoagulanlar da nonspesifik inhibitör şeklinde tanımlanabilirler (8).

Karışım (Mixing) Çalışmaları

Uzamış aPTZ sonucunun sebeplerini araştırmadan önce örneğin heparin ile kontaminasyonu araştırılmalıdır. Hastada trombolitik ajan, vitamin K antagonisti ile trombin inhibitörü kullanımı sorgulanmalıdır. Ayrıca örnek içeriği kontrol edilmeli pıhtı, hemoliz, ikter, lipemi varlığına bakılmalıdır. Tüp doluluk miktarı kontrol edilmeli ve hematokrit düzeyi >%55 olan örneklerde tüpteki sitrat miktarı hesaplanmalı ve bu sitrat miktarıyla alınmış yeni örnek ile aPTZ çalışılmalıdır (6).

Altta yatan nedeni belirlemek için, uzamış aPTZ sergileyen numuneler üzerinde bir karışım çalışması yapılmalıdır. Bu çalışma, hastanın plazmasının 1:1 hacim/hacim oranında NPP ile karıştırılmasını içerir. Clinical Laboratory Standards Institude (CLSI) kurallarına göre NPP'ler en az 20 sağlıklı kişiden hazırlanmalı veya ticari bir kuruluştan temin edilmelidir. NPP, tüm faktörleri yaklaşık %100 oranında içermeli, aPTZ sonucu referans ortalamasına yakın ve taze dondurulmuş olmalıdır (6).

Karışım işlemi sonrasında aPTZ yeniden ölçüldüğünde, sonuç referans aralığına yaklaşır ise zamana bağımlı olarak etki eden sıklıkla FVIII inhibitörleri ve LA'ları (%15'i zamana bağımlı) atlamamak için yeni hazırlanacak numuneler 37° C'de 1-2 saat süreyle inkübe edilmelidir. Bu işlem, Hasta-NPP karışımı (1:1), hasta plazması ve NPP olmak üzere 3 örnek şeklinde hazırlanır ve kapakları kapalı bir şekilde 37 °C'de 1-2 saat inkübe edilir. İnkübasyon periyodu sonrası inkübe edilmiş NPP ile inkübe edilmiş hasta plazması 1:1 oranında karıştırılarak yeni bir karışım elde edilir. Bu kontrol tüpü sayesinde inkübasyon periyodu süresinde gelişebilecek FV ve FVIII kaybı ekarte edilmiş olur. İnkübe Hasta-NPP karışımı ile yeni kontrol karışımında aPTZ ölçülür. Eğer inkübe karışım örneği ile kontrol karışım örneği aPTZ sonuçları eşit ve aPTZ referans aralığına yaklaşır ise faktör eksikliği düşünülür. Eğer inkübasyonlu karışım aPTZ sonucu kontrol karışım örneğinden uzun ise zaman bağımlı inhibitör varlığından şüphenilmelidir. Hastanın klinik hikayesinin bilinmesi LA veya spesifik inhibitörlerin taranması gibi daha ileri tetkikler için yol gösterici olacaktır (6).

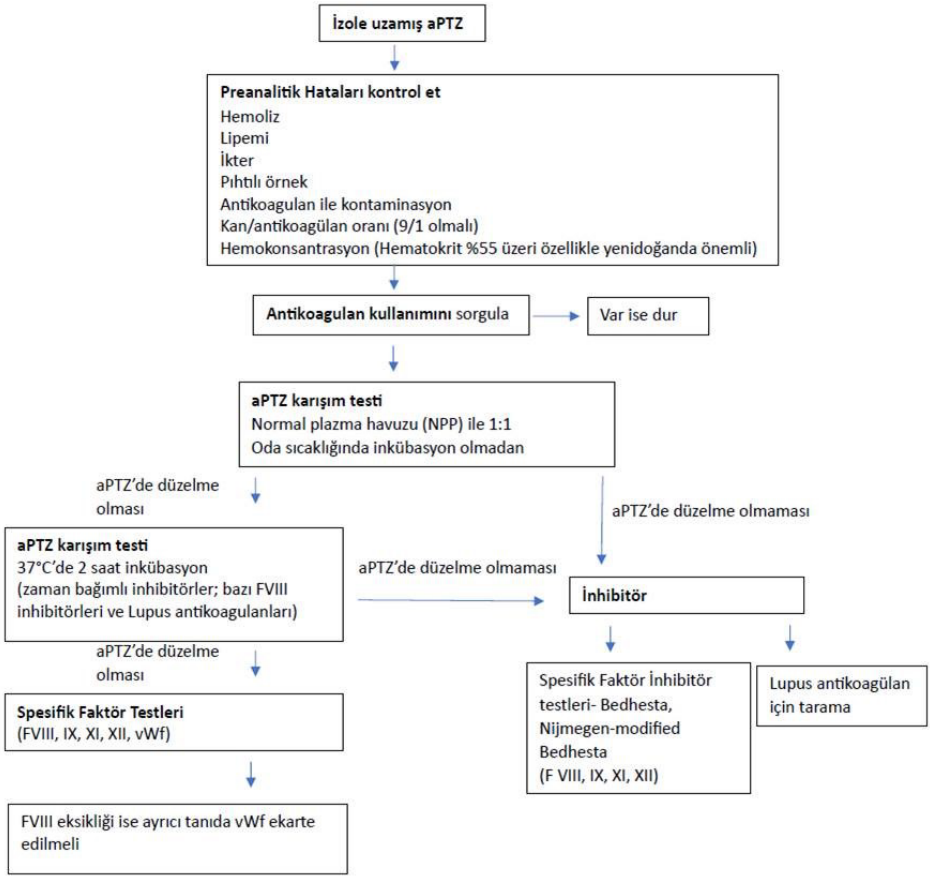
Karışım testlerinin yorumlanmasında Rosner indeksi ve Yüzde Düzeltme formülü de kullanılabilir (27,28). (Şekil 1). Ayrıca inkübasyonlu karışım tüpü aPTZ sonucu ile kontrol tüpü aPTZ arasındaki fark %10'dan fazla ise zamana bağımlı inhibitör pozitif lehine değerlendirilebilir (12).

Bazı çalışmalar, 4:1 (hasta plazması: NPP) karışımındaki yüzde düzelmenin, antikoagülan ve faktör eksikliklerini tanımlamak için 1:1 karışıma göre daha iyi duyarlılık ve özgüllük sağladığını göstermiştir (28).

Spesifik faktör inhibitör testleri

Spesifik faktör inhibitörlerin tespiti ve miktarının belirlenmesi, ELISA gibi antijen-antikor analizleri veya Nijmegen ve Bedhesda analizleri gibi fonksiyonel ve pıhtılaşma temelli yöntemler kullanılarak gerçekleştirile-

bilir. İnhibitör seviyeleri Bethesda birimleri (BU) cinsinden ifade edilir; bir BU, 37°C'de 2 saatlik inkübasyonun ardından plazmadaki FVIII aktivitesinin %50'sini nötralize etmek için gerekli olan FVIII inhibitörünün miktarını temsil eder. Pozitif sonuç, FVIII için >0.6 BU, FIX için ise >0.3 BU'tir. İnhibitör testi için endikasyonlar Tablo 4'te belirtilmiştir.



Şekil 1. İzole uzamış aPTZ'de karışım testi algoritması

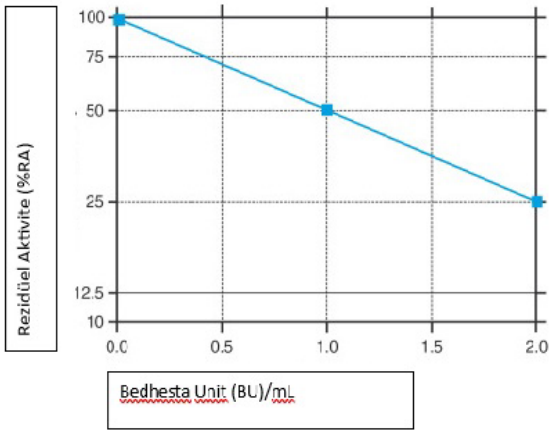
Figure 1. Mixing Test Algorithm in Isolated Prolonged aPTT.

Tablo 4. İnhibitör Testi için Endikasyonlar
Table 4. Indications for Inhibitor Test.

İnhibitör Testi İsteminde Endikasyonlar
Başlangıç faktör replasman tedavisi sonrası
Yoğun faktör replasman tedavisi sonrası; 5 günden fazla her gün tedavi alımı …
Yeterli faktör replasman tedavisine rağmen tekrarlayan kanama veya eklem içi kanama
Faktör replasman tedavisine yanıt alınamaması
Faktör replasman tedavisi sonrası beklenenden daha düşük yarılanma ömrü
Faktör replasman tedavisi sonrası optimal olmayan klinik veya laboratuvar yanıt
Faktör replasman tedavisine ameliyat sonrası istenen yanıtın alınamaması
Ameliyat öncesi

Bedhesda Testi

Hasta plazması imidazol tamponlu NPP ile bir seri dilüe edilir. Kontrol olarak, imidazol tamponlu NPP, faktör eksik plazma ile 1:1 dilüe edilir. 2 saat 37°C inkübasyondan sonra (FIX için inkübasyona gerek yok) dilüe karışımlardaki faktör aktiviteleri ölçülür ve kontrol reaksiyonununkine göre (%) hesaplanır. Rezidüel aktivite (RA) hesabı =hasta karışımı faktör aktivitesi/kontrol karışımın faktör aktivitesi*100 olarak hesaplanır. Geçerli RA'ların tanımlanmış aralığı %75'ten %25'e kadar sınırlıdır, bu nedenle %50'ye en yakın RA seçilir (12,29). RA sonucunu Bedhesda birimiyle ilişkilendiren grafik inşa edilmiştir (Şekil 2). Bu grafikten BU/mL bulunur ve nihayi sonuç dilüsyon oranı ile çarpılarak elde edilir. Örneğin RA sonucu 60 olan bir örneğin grafikten 0.74 BU/mL olduğu bilinmektedir. Eğer RA değeri 1/8 dilüsyondan elde edildi ise sonuç 0,74*8=5.92 BU/mL olarak verilecektir.



Şekil 2. Rezidüel Aktivite (RA) sonucu ile Bedhesda birimi ilişkisi

Figure 2. Relationship Between Residual Activity (RA) Result and Bethesda Unit.

İnhibitörlerin laboratuvar testlerindeki etkileri aynı değildir. Çoğu inhibitör (Tip 1) artan dilüsyonlarla rezidü FVIII aktivitesinde artış gösterirken bazı inhibitörler ise bu artışı göstermez (Tip 2). Tip 2 inhibitörlerinin kalibrasyon eğrisine paralelliği yoktur bu nedenle titre edilmesi zor olabilir. Bunun yanında bazı FVIII inhibitörleri, FVIII klirensini arttırıp FVIIII aktivitesini etkilemez. Bu nötrleştirici olmayan FVIII inhibitörleri karışım testleri ile tespit edilemez ve Bedhesta testlerinde saptanamaz. ELISA testleri bu tür nötrleştirici olmayan FVIII inhibitörlerini saptamada kullanılabilir (12).

Nijmegen Modifiye Bedhesda Testi

Nijmegen modifiye bedhesda testi, analitik tespit sınırına yakın sonucu olan örneklerde özgüllüğü arttırır. Nijmegen modifikasyonları, dilüsyonda kullanılan imidazole tamponunun faktör eksik plazma ile değiştirilmesi ve NPP'nın 7.4'e tamponlanması ile gerçekleştirilir. Bu değişiklikler sayesinde FVIII'in inkübasyon sırasında kaybının azalması önlenmiş olur. NPP tamponlanması sayesinde pH artışı önlenir ve faktör eksik plazma ile dilüsyonların yapılması örneklerin içindeki diğer protein konsantrasyonlarının azalmasını önler (12). Böylece Nijmegen modifikasyonu, normal plazmayı tamponlayarak ve seyreltici tamponu FVIII'den yoksun plazmayla değiştirerek testin duyarlılığı ve özgüllüğünü artırmış olur (4). WFH Hemofili hastalarında inhibitör izleminde Nijmegen modifiye bedhesda testinin çalışılmasını önerir (2).

SONUÇ

Farklı kanama bozuklukları çok benzer semptomlara sahip olabilir; bu nedenle, hastanın uygun tedaviyi almasını sağlamak için doğru tanı şarttır. Hemofili hastalarında doğru tanı ancak kapsamlı ve güvenilir laboratuvar hizmeti ve uzman desteği ile yapılabilir. Bu durum laboratuvarda uygun protokol ve prosedürleri takip ederek, pıhtılaşma laboratuvar testlerinde bilgi ve uzmanlık sahibi olunarak, doğru ekipman ve reaktiflerin kullanılması ve kalite güvencesi ile sağlanabilir. Hemofili hastalarında uygun tanı ve izleminin uygulanabilmesi laboratuvar uzmanı ile klinisyenin sürekli iletişim halinde olması ile gerçekleştirilebilir.

Conflict of Interest: None

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Olgu Sunumu / *Case Report*

Prolactin Elevation Due to Heterophile Antibody Interference in Pituitary Deficiency

Hipofiz Yetmezliğinde Heterofil Antikor İnterferansı Nedeniyle Prolaktin Yüksekliği

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ABSTRACT

This case highlights the effect of heterophilic antibody interference in two siblings with hypopituitarism. Prolactin measurements performed on the UniCel DxI 800 in patients followed up with a diagnosis of hypopituitarism were found to be falsely elevated. For further investigation, polyethylene glycol precipitation (PEG), heterophilic antibody blocking tubes (HBT), serial dilution, and three different blocking antibodies were used. The PEG test showed a reduction of less than 40%, and macroprolactinemia was excluded. In the serial dilution test, the results were not linear, raising suspicion of interference. Lower prolactin levels were reported in three different systems. After HBT application, prolactin levels unexpectedly increased in both siblings. The increase in prolactin levels observed after HBT and the addition of different blocking antibodies could be due to an unexpected interaction between the blocking antibodies and the analysis reagents. The investigations using blocking antibodies were unable to determine the exact nature of the interfering agent.

Key Words: Prolactin, Hyperprolactinemia, Heterophil Antibodies, Interference, Hypopituitarism

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ÖZET

Bu vaka, hipopituiterizmli iki kardeşte heterofilik antikor interferansının etkisini vurgulamaktadır. Hipopituiterizm tanısı ile takip edilen hastalarda UniCel DxI 800 üzerinde yapılan prolaktin ölçümleri yanlış şekilde yüksek bulunmuştur. İleri inceleme için polietilen glikol çökelmesi (PEG), heterofilik antikor engelleyici tüpler (HBT), seri dilüsyon ve üç farklı blokan antikor kullanılmıştır. PEG testi, % 40'ın altında bir azalma göstermiş ve makroprolaktinemi dışlanmıştır. Seri dilüsyon testinde, sonuçların doğrusal olmaması nedeniyle interferans şüphesi ortaya çıkmıştır. Üç farklı sistemde ise daha düşük prolaktin seviyeleri raporlanmıştır. HBT uygulaması sonrası prolaktin seviyeleri her iki kardeşte de beklenmedik şekilde artmıştır. Farklı bloke edici antikorların eklenmesinden sonra da prolaktin sonuçlarında görülen artış, bloke edici antikorlar ile analiz reaktifleri arasındaki beklenmedik etkileşimden kaynaklanabilir. Blokan antikorlarla yapılan incelemeler interferans ajanının tam doğasını belirleyememiştir.

Anahtar Sözcükler: Prolaktin, Hiperprolaktinemi, Heterofil Antikorlar, İnterferans, Hipopitüitarizm

INTRODUCTION

Hormone immunoassays can be significantly influenced by the presence of heterophile antibodies, human anti-animal antibodies, autoantibodies, and rheumatoid factors, which can lead to inaccurate hormone measurements. These interferences compromise assay precision by interacting with antibodies or detection labels, such as streptavidin or alkaline phosphatase (ALP), used in the reagents. In enzyme-based immunoassays, the presence of enzyme inhibitors or activators can also alter the signal, resulting in altered test results (1-4). Hypopituitarism is a rare condition characterized by a deficiency in one or more hormones produced by the pituitary gland. The most common cause of hypopituitarism is pituitary tumors, which account for 61% of cases. These tumors may lead to the overproduction of a specific hormone while simultaneously causing deficiencies in others. Hypopituitarism is associated with an increased risk of mortality, particularly due to higher rates of cardiovascular and respiratory diseases, underscoring the importance of early diagnosis and intervention. Accurate hormone measurements are especially critical in managing conditions like hypopituitarism, where precise hormone monitoring is essential (5-7). Here, we present the case of two siblings undergoing hormone assessments for hypopituitarism, where interference in the assays resulted in inconsistent prolactin levels across different immunoassay platforms.

CASE PRESENTATION

The two siblings, aged 18 (patient 1) and 11 (patient 2), have been receiving growth hormone (GH) replacement therapy for the past three years due to hypopituitarism. During this period, their prolactin levels were measured seven and nine times, respectively, using the Infinity c 8000 analyzer (Roche Diagnostics, Penzberg, Germany), consistently showing low results (<0.094 ng/mL for both). Additionally, both siblings exhibited low GH responses following the Clonidine GH Stimulation Test. GH levels, measured on the same Infinity c 8000 analyzer, did not exceed 7 ng/mL, confirming GH deficiency (8). However, when testing was switched to the UniCel DxI 800 (Beckman Coulter, Brea, CA, USA), significantly elevated prolactin levels were observed in both siblings: 41.22 ng/mL for patient 1 and 27.72 ng/mL for patient 2. These high prolactin values were inconsistent with both the previous results and the clinical diagnosis, prompting further investigation. Informed consent was obtained to participate in the study and a new sample was drawn for re-evaluation

Suspecting the presence of heterophile antibodies, further studies were conducted. The patients were called and invited to the

laboratory. Prolactin levels were re-measured on the UniCel DxI 800 analyzer. The detection limit of prolactin assay was 0.25 ng/mL, and the total coefficient of variation (CV) was 6.92%, 3.32%, and 4.23% for low, medium, and high prolactin concentrations, respectively. (catalog number: 472010). To remove potential interfering antibodies, the patients' sera underwent precipitation with polyethylene glycol (PEG) 6000 (product number: 29577, Merck Ltd.). Simultaneous analyses of both the control sample and the patients' sera were performed, and the percentage decrease in prolactin levels after PEG precipitation was calculated. The percentage decreases were below 40% for both and macroprolactinemia was excluded (Table 1). For further investigation of heterophile antibodies, heterophilic blocking tubes (HBT) (Scantibodies Laboratory, Inc., USA, catalog number:0257C) were utilized, comparing results with the original measurements. The HBT contains specific binders that deactivate heterophile antibodies in 500 µL of sample. Analyses were carried out simultaneously on a control sample and the patients' sera. A percentage change of 9.67 % was found for the control, which was acceptable, whereas prolactin levels were increased by 320.3% and 94.10% in patient 1 and patient 2 respectively. (Table 1) Dilution testing was conducted through serial dilutions (1/2, 1/4, 1/8, 1/16, and 1/32) using the manufacturer's zero calibrator and results showed the presence of interferences since the test was not linear (Table 2). Subsequently, samples were sent to other laboratories using the Advia Centaur XP (Siemens Healthcare Diagnostics, Tarrytown, USA), Alinity i 1000 (Abbott Laboratories, Diagnostic Division, Abbott Park, IL, USA), and Roche systems. All platforms returned low prolactin results consistent with the initial measurements from the Roche Infinity c 8000 analyzer. Additional tests, including ALP, rheumatoid factor (RF) and immunofixation analyses, showed that ALP and RF levels remained within the reference range, and no monoclonal peaks were found on serum immunofixation analysis. Further investigation was performed at the Beckman Coulter complaint handling unit laboratory, where three different blocking reagents were applied to the patients' sera. Pools 1 and 2 contained different blockers: PolyMak 33 and HBR-1 (Pool 1), and Goat, Mouse, Rabbit, Sheep, and Bovine IgGs (Pool 2), all animal-derived antibodies. Pool 3 (AP Mutein, Scavenger ALP), a pool of blocker related to ALP, was also tested. The percent changes in prolactin levels for patient samples ranged between -10.00% and +37.99% after the addition of the pools 1/2/3. A percent change between -25% and +25% is expected if no interference is present. For patient 2, percent changes above +25% were observed with Pools 1 and 3, suggesting that the blockers were ineffective in decreasing the prolactin results (Table 3). Since the changes were positive, these results could not be interpreted. The interference testing did not allow for the identification of the nature of the interference, as none of the blockers used decreased the signal, and other potential interfering substances may exist.

Table 1. PEG Precipitation and HBT Analysis of Patients and Control
Tablo 1. Hastaların ve Kontrollerin PEG Presipitasyonu ve HBT Analizi

	Neat Results (ng/mL)	PEG Results (ng/mL)	Percentage Change (%)	HBT Tube (ng/mL)	Percentage Change (%)
Patient 1	66.32	50.50	-23.85	128.73	+94.10
Patient 2	34.88	33.98	-2.58	146.61	+320.3
Control	6.23	4.93	-20.86	10.34	+9.67

Table 2. Serial Dilution Results of The Patients
Tablo 2. Hastaların Seri Seyreltme Sonuçları

	Dilution	Prolactin Results (ng/mL)	Percent Change (%)
Patient 1	Neat	66.32	-
	1:2 Dilution	62.45	-5.83
	1:4 Dilution	52.85	-20.28
	1:8 Dilution	40.98	-38.20
	1:16 Dilution	33.67	-49.23
	1:32 Dilution	30.83	-53.51
Patient 2	Neat	34.88	-
	1:2 Dilution	32.48	-6.88
	1:4 Dilution	28.12	-19.38
	1:8 Dilution	20.33	-41.71
	1:16 Dilution	18.02	-48.33
	1:32 Dilution	17.71	-49.22

Table 3. Observed Changes Using Pool 1, Pool 2, and Pool 3
Tablo 3. Havuz 1, Havuz 2 ve Havuz 3 Kullanılarak Gözlemlenen Değişiklikler

	Prolactin Results (ng/mL)	Percent Change (%)
Patient 1 (Neat)	56.76	-
Patient 1 + Pool 1	52.46	-7.57
Patient 1 + Pool 2	51.36	-9.51
Patient 1 + Pool 3	63.08	+11.13
Patient 2 (Neat)	30.78	-
Patient 2 + Pool 1	42.48	+37.99
Patient 2 + Pool 2	27.70	-10.00
Patient 2 + Pool 3	39.44	+28.12

Pool 1: PolyMak 33 and HBR-1,
Pool 2: Goat, Mouse, Rabbit, Sheep and Bovine IgGs,
Pool 3: AP Mutein, Scavenger ALP

DISCUSSION

This case underscores the significant impact of heterophile antibody interference on hormone immunoassays. In the presented cases of the two siblings, the presence of heterophile antibodies resulted in falsely elevated prolactin levels when tested on the UniCel DxI 800 analyzer while other platforms showed low levels. A comprehensive investigation, including PEG precipitation, serial dilution, and HBT tests were performed to confirm the presence of heterophile antibodies. However, analyses using different blocking antibodies could not allow for the identification of the nature of the interference.

Over the years, various interferences in immunoassays have been identified. While some of these interferences are now rarely encountered in routine practice, issues such as cross-reactions, heterophile antibodies, biotin, and anti-analyte antibodies continue to pose challenges. Additionally, as new therapies are developed, new types of interference are emerging adding complexity to immunoassay evaluations. The interference may have been caused by an exogenous substance, such as a drug or compound absorbed by the patients, or an endogenous factor, such as heterophile or anti-animal antibodies produced by the patients (9). Both siblings, undergoing growth hormone replacement therapy with

daily doses of recombinant human growth hormone (rhGH, Omnitrope, Genotropin) between 0.7 and 1.5 mg. According to the Beckman Coulter. Access Prolactin Reagent Kit Insert. it is stated: "No significant cross-reactivity was observed when recombinant human growth hormone (rhGH) was added to the Access Prolactin Calibrator S1 (2 ng/mL) at 10.82 IU/L." Given that the administered rhGH doses were lower than this concentration, interference related to rhGH treatment was considered unlikely.. Despite advances in our knowledge and understanding of the mechanisms of interference in immunoassays, there is no single procedure that can rule out all interferences (10). Prolactin tests generally use the sandwich immunoassay principle, but they differ in the technologies used for labeling. The Cobas 8000 system uses Electrochemiluminescence Immunoassay (ECLIA), where biotinylated and ruthenium-labeled prolactin-specific antibodies are used to form a sandwich complex UniCel DxI 800 system is a one-step sandwich chemiluminescent immunoassay using paramagnetic particles. It utilizes a polyclonal goat anti-PRL alkaline phosphatase conjugate and paramagnetic particles coated with a Mouse monoclonal anti-PRL antibody. The Alinity i 1000 system is a two-step automated test using Chemiluminescent Microparticle Immunoassay (CMIA) technology, where prolactin binds to anti-prolactin-coated microparticles, and an acridinium-labeled conjugate is added afterward. The Centaur XP Prolactin assay is a 2-site sandwich immunoassay using direct chemiluminescent technology. It involves two fixed antibodies: a goat polyclonal anti-prolactin antibody labeled with acridinium ester in the reagent, and a mouse monoclonal anti-prolactin antibody attached to paramagnetic particles in the Solid Phase. Several studies have identified alkaline phosphatase (ALP) as a potential source of interference in immunoassays (11-12). In a study by Herman et al., elevated ALP levels (>1000 U/L) were found to interfere with assays like DxIcTnI and hCG. Yıldız et al. reported a case of falsely low unconjugated estriol (uE3) levels in a 35-year-old pregnant woman due to assay interference. Initial screening on the UniCel DxI 800 analyzer indicated a high risk for Down syndrome, but re-testing on the IMMULITE 2000 XPi showed a normal result. Further investigations revealed that heterophile antibodies and alkaline phosphatase scavenger increased uE3 levels, confirming interference (12). The researchers stressed the importance of evaluating the effect of ALP interference during the method validation process, particularly in immunoassays that rely on ALP for signal amplification. In our study, adding Pool 3, which contains Scavenger ALP (a blocker related to ALP), did not eliminate the interference leading us to conclude that the interference was not related to ALP.

In conclusion, this case highlights the critical need for collaboration between laboratory professionals, clinicians, and assay manufacturers to identify and address assay interferences. Despite extensive investigations, the exact nature of the interfering agent could not be determined, emphasizing the challenges in resolving immunoassay interference. Ongoing education, research into underlying mechanisms, and improved strategies for managing interferences are essential for ensuring accurate testing and optimal patient care, particularly in conditions where hormone level fluctuations significantly impact treatment. Future efforts should focus on the identification of new interferences and developing strategies to minimize their impact on clinical practice, with manufacturers playing a key role in this process.

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