

## Interferencije svojstvene kvantitativnim imunokemijskim metodama

### Interferences in quantitative immunochemical methods

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#### Sažetak

Antitijelo koje se u imunokemijskim metodama koristi kao reagens otkriva ciljni analit (antigen). Iako je nekovalentna veza između analita i komplementarnog antitijela specifična, moguće su lažno pozitivne ili negativne interferencije. Neke su interferencije slične interferencijama kod kemijskih analiza, a neke su svojstvene samo imunokemijskim analizama. Na interferencije u imunokemijskim metodama treba pomisliti ako se dobije neprihvatljiv rezultat, ako postoji nelinearnost prilikom razrjeđivanja, ako nema podudarnosti s ostalim nalazima, odnosno kliničkim podacima, ako se različitim imunokemijskim metodama dobiju značajno različiti rezultati određivanja istog analita. U ovom će radu biti opisane neke od mogućih interferencija: 1) križna reaktivnost s endogenim i egzogenim substancijama koje nemaju strukturu antitijela; 2) križna reaktivnost s endogenim i egzogenim supstancama koje imaju strukturu antitijela; 3) prozonski učinak – *hook* efekt; i 4) utjecaj matriksa. Poznavanjem i prepoznavanjem interferencija u imunokemijskim analizama mogu se izbjeći moguće neželjene posljedice: pogreške u dijagnozi, liječenju i praćenju uspješnosti liječenja, nepotrebna dodatna laboratorijska istraživanja, nepotrebna terapija.

**Ključne riječi:** imunokemijske metode; interferencije; križna reaktivnost; prozonski učinak

#### Abstract

In the immunoassays, an antibody used as a reagent, detects an analyte (antigen) of interest. Although the noncovalent bound between analyte and complementary antibody is specific, false-positive and false-negative interferences are possible. Some interferences are similar to those in chemical analyses and some are typical only for immunoassays. One should suspect interferences in following cases: upon receiving an unacceptable result, if there is non-linearity during dilution, if there is no agreement with other test results or clinical data, if different immunoassays in determination of the same analyte provide significantly different results. This paper reviews some of the possible interferences: 1) cross-reactivity with endogenous and exogenous non antibody-structured substances; 2) cross-reactivity with endogenous and exogenous antibody-structure substances; 3) the hook effect; and 4) the matrix effect. By knowing and recognizing interferences in immunoassays, one can avoid possible undesired consequences: diagnostic errors, treatment and monitoring of its efficacy, unnecessary additional laboratory testing, unnecessary therapy.

**Key words:** immunoassays; interferences; cross-reactivity; prozone effect

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#### Uvod

Razvoj imunokemijskih metoda, osobito zadnjih tridesetak godina, revolucionizirao je laboratorijsku medicinu. Primjena novih biljega završne reakcije, novih oblika testova, automatizacija, ponovljivost, brzina izvođenja i dos-

#### Introduction

The development of immunoassays has revolutionized laboratory medicine, especially during the last 30 years. The implementation of new endpoint tracers, new assay formats, automation, reproducibility, duration time of

tupnost analiza pridonijeli su da imunokemijske metode postanu svakodnevna praksa. Osnovno svojstvo svih imunokemijskih metoda – od imunoprecipitacijskih do biočip metoda – jest da reagens, kojim se otkriva ili kvantitativno određuje ciljni analit (antigen), sadrži antitijelo. Iako je nekovalentna veza između analita i komplementarnog antitijela specifična, moguće su brojne interferencije (Slika 1.) koje uzrokuju dobivanje lažno povećanih (pozitivna interferencija) (1-3) ili lažno smanjenih rezultata (negativna interferencija) (4,5). U svakodnevnom radu nužno je misliti na uvijek prisutne predvidljive i uvijek moguće nepredvidljive i neprepoznatljive interferencije (6). Jedan od najdrastičnijih primjera pogreške u medicinskoj praksi jest primjer lažno-pozitivnog nalaza humanog korionskog gonadotropina (hCG), opisan kod 22-godišnje žene koja je, zbog neprepoznate interferencija heterofilnih antitijela te stoga trajno lažno-pozitivnog nalaza hCG, podvrgnuta nepotrebnim medicinskim zahvatima: kemoterapiji, histerektomiji i segmentalnoj plućnoj resekciji (7). Taj je slučaj dobio pozornost javnih glasila (odšteta 16 milijuna USD). Međutim, stručno-znanstvena publicistika opisuje slične slučajeve (8,9).

Interferencije bi se mogle definirati kao učinak tvari prisutnih u analitičkom sustavu koje uzrokuje promjenu izmjerene vrijednosti u odnosu na pravu vrijednost (10). Neke su interferencije svojstvene imunokemijskim metodama. Posebne preanalitičke i analitičke interferencije utječu na kliničko vrednovanje imunokemijskih nalaza u usporedbi s ostalim kemijskim analizama. Zbog osobitih svojstava imunokemijskih metoda (križna reaktivnost antitijela, specifičnost, ograničenja analitičke osjetljivosti, utjecaj matriksa, itd.) može doći do nesklada laboratorijskih nalaza.

Proizvođači reagensa za imunokemijske analize dužni su upozoriti na te interferencije, a neke su obično naznačene u uputama za izvođenje analitičkog postupka. Interferencije koje ovise o analitu odnose se na interakciju tvari prisutnih u biološkom uzorku i jedne (1,11-16) ili više komponenata iz reagensa (17,18). Učinak interferencije obično ovisi o koncentraciji interferenta (12,19). Interferencije koje ovise o analitu podrazumijevaju spojeve koji su strukturno slični analitu. Zbog toga ti spojevi reagiraju križno s antitijelom, ili ostalim proteinima u uzorku, primjerice autoantitijelima prema analitu, heterofilnim antitijelima, humanim anti-animalnim antitijelima (12). Najčešće opisivane interferencije su one pri određivanju hormona (5,20-24), tumorskih biljega (25), lijekova i metabolita (26,27), troponina (29-31), te pri serološkim analizama (32-33). Opisano je da antitijela prema analitu (autoantitijela) uzrokuju interferencije za brojne analite, primjerice za tiroidne hormone (ukupne i slobodne oblike), tireoglobulin, prolaktin (makroprolaktinemija može imati kao posljedicu hiperprolaktinemiju, a da ne postoji bolesti hipofize), testosteron, izulin (12). Antitiroidna autoantitijela su

assay and availability of analyses have contributed for immunoassays to become everyday practice. The main characteristic of all immunoassays – from immunoprecipitation to biochip assays – is that the reagent that discovers or quantifies the target analyte (antigen) contains the antibody. Despite the specificity of the noncovalent bond between analyte and complementary antibody, numerous interferences (Figure 1) are possible, and can cause false increase (positive interference) (1-3) or false decrease of measured result (negative interference) (4,5). In every research, it is necessary to think about always present predictable and always possible unpredictable and unrecognizable interferences (6). One of the most drastic examples of error in medical practice is the case of false positive chorionic gonadotropin (hCG) test result, described with 22 year old women who underwent, due to unrecognized interference of heterophilic antibodies followed by permanent false positive hCG test result, unnecessary medical interventions: chemotherapy, hysterectomy and segmental lungs resection (7). That case won a lot of public attention (16 million USD damage was paid). Moreover, scientific literature offers similar cases (8,9).

Interferences may be defined as the effect of substances present in an analytical system which causes deviation of the measured value from the true value (10). Some interferences are typical for immunochemical methods (11). The special pre-analytical and analytical interferences have their impacts on the clinical evaluation of immunochemical findings, compared to other chemistry methodologies. Due to the specific features of the immunoassay technique (cross-reactivity of the antibodies, specificity, technology-dependent sensitivity limits, the matrix effect, etc.), which might cause misleading laboratory report. Manufacturers of reagents for immunoassays are obliged to warn against these interferences, and some of them can usually be found in instructions for analytic procedure. Analyte-dependent interferences relate to interaction of substances present in biological sample and one (1,11-16) or more reagent components (17,18). The interference effect usually depends on concentration of interfering substance (12,19). These analyte-dependent interferences include compounds that are structurally similar with analyte. Therefore, they cross-react with the antibody or other proteins in the sample, e.g. autoanalyte antibodies, heterophile antibodies, human anti-animal antibodies or rheumatoid factors (12). The most frequently described interferences are the ones occurring during hormone determination (5,20-24), tumor markers (25), drugs and metabolites (26-28.), troponin (29-31) and during serological analyses (32,33). Autoanalyte antibodies (autoantibodies) have been described to cause interferences for a number of analytes, e.g. thyroid hormones (total and free forms), thyroglobulin, prolactin (macroprolactinaemia can result in hyperprolactinaemia without pituitary

nađena u bolesnika s Gravesovom bolesti, Hashimotovim tireoiditisom, hipertireoidizmom nakon liječenja, u bolesnika s gušom, karcinomom ili ne-tireoidnim autoimunim stanjima. Prisustvo reumatodnog faktora u serumu može uzrokovati lažno povećane koncentracije troponina, kao i interferirati kod ispitivanja funkcije štitnjače. Zbog steričkog blokiranja reakcije analit-antitijelo, paraprotein može također interferirati u imunokemijskim analizama.

Na interferencije u imunokemijskim metodama treba pomisliti ako se dobije neprihvatljiv rezultat, ako postoji nelinearnost prilikom razrjeđivanja, ako nema podudarnosti s ostalim nalazima, odnosno kliničkim podacima, ako se različitim imunokemijskim metodama dobiju značajno različiti rezultati određivanja istog analita. Nepoznavanje i neprepoznavanje interferencija može imati kao posljedicu pogreške u dijagnozi, liječenju i praćenju uspješnosti liječenja, nepotrebna dodatna laboratorijska istraživanja, nepotrebnu terapiju (kod lažno smanjene koncentracije analita bolesnik se može predozirati). Većina je interferencija svojstvena svim oblicima imunokemijskih metoda (Tablica 1.), a neke se interferencije odnose na pojedine metode.

Interferirajuća antitijela mogu utjecati na sve vrste imunokemijskih analiza, ali su najčešća u saturacijskim analizama. Razlog tome jest taj što saturacijske analize podrazumijevaju suvišak obaju antitijela (primarnog i obilježenog). Njihova je koncentracija veća od uobičajene koncentracije analita, pa se reakcija odvija vrlo brzo u uvjetima velike analitičke osjetljivosti. Bilo koje antitijelo iz seruma koje ima i neznatan afinitet prema primarnom i obilježenom antitijelu može s njima stvarati mjerljivi kompleks. Sva antitijela seruma koja su dovoljno velika da mogu vezati istodobno dva antitijela iz reagensa, na kraju daju mjerljivi signal. Jedan od čestih primjera interferencija su idiotipska antitijela, primjerice reumatodini faktori koji sadrže križno reaktivne idioptope (25).

U ovom će radu biti bliže opisane neke od mogućih interferencija svojstvenih imunokemijskim metodama, poglavito one koje bi klinički biokemičar morao poznavati: 1) križna reaktivnost s endogenim i egzogenim supstancama koje nemaju strukturu antitijela; 2) križna reak-

disease), testosterone, insulin (12). Antithyroid autoantibodies have been found in patients with Graves' disease, Hashimoto's thyroiditis, hyperthyroidism after treatment, goiter, carcinoma or non-thyroid autoimmune conditions. The presence of rheumatoid factors in serum can cause falsely elevated values in troponin assays as well as in thyroid function methods. Also, paraprotein can interfere in immunoassay by sterical blocking analyte-antibody reaction.

One should suspect interferences in immunoassays upon receiving an unacceptable result, if there is non-linearity during dilution, if there is no agreement with other test results or clinical data, if different immunoassays in determination of the same analyte provide significantly different results. Unawareness and non-recognition of interferences could lead to diagnostic errors, inadequate treatment and monitoring of its efficacy, unnecessary laboratory tests, unnecessary therapy (falsely low analyte concentration can lead to patient overdose). Most of the interferences are typical for all immunoassays (Table 1) and some relate to single methods.

Interfering antibodies can affect all types of immunochemical analyses, but they are most frequently present in saturating analyses. That is because in saturating analyses we have an excess of both antibodies (the capture and the tracer one). Their concentration is higher than the usual analyte concentration and the reaction occurs very fast in conditions of high analytic sensitivity. Any serum antibody having only slight affinity to the capture and the tracer antibody can together with them create a measurable complex. All serum antibodies big enough to bind simultaneously two antibodies from the reagent in the endpoint, provide a measurable signal. One of the common examples of interfering substances are idiotopic antibodies, e.g. rheumatoid factors containing cross-reactive idiotopes (25).

This paper gives a close review of some of the possible interferences typical for immunoassays, especially of those of greater importance for a clinical biochemist: 1) cross-reactivity with endogenous and exogenous non antibody-structured substances; 2) cross-reactivity with endo-

**TABLICA 1.** Interferencije kod pojedinih imunokemijskih metoda

Interference	Methods
Cross-reactivity	All, but mostly competitive
Prozone effect (hook effect)	Nephelometric, turbidimetric, saturating
Matrix	All
Antibodies	All, but mostly saturating

**TABLE 1.** Interferences in particular immunoassays

tivnost s endogenim i egzogenim supstancama koje imaju strukturu antitijela; 3) prozonski učinak - hook efekt; i 4) utjecaj matriksa.

### Križna reaktivnost s endogenim i egzogenim supstancama koje nemaju strukturu antitijela

Križna je reaktivnost najčešća interferencija u imunokemiji, ali najčešće u kompetitivnim metodama. Radi se o nespecifičnom utjecaju tvari u uzorku, koja je strukturalno slična analitu (ima jednake ili slične epitope kao analit) te nadmeće se za vezno mjesto na antitijelu (34). Stupanj interferencije uzrokovane križnom reaktivnošću ovisi o tri čimbenika: specifičnosti antitijela, obliku testa i pripremi uzorka (35). Najčešći su primjeri prilikom određivanja koncentracije hormona, lijekova, specifičnog IgE prema alergenima. Hormoni TSH (tireotropin, engl. *thyroid-stimulating hormone*), LH (luteinizirajući hormon; engl. *luteinizing hormone*), FSH (folikulo-stimulirajući hormon; engl. *follicle-stimulating hormone*) i hCG (humani korionski gonadotropin; engl. *human chorionic gonadotropin*) imaju analogan  $\alpha$ -lanac, a  $\beta$ -lanac određuje specifičnost pojedinog hormona – stoga treba odabrati metodu koja će specifičnim antitijelima moći prepoznati različite epitope (36). Drugi je primjer steroidnih hormona, koji imaju jednaku ciklopentanoperhidrofenantrensku strukturu (37,38). Nadalje, prostatični specifični antigen (engl. *prostate specific antigen*, PSA) – postoji u nekoliko oblika [ukupni PSA, slobodni fPSA, prekursor proPSA, a u novije vrijeme i antigen ranog rasta karcinoma prostate (engl. *early prostate cancer antigen*, EPCA)], koji su uzrok križnoj reaktivnosti i nedovoljnoj točnosti (39). Križna reaktivnost uzrokuje lažno povećane vrijednosti ispitivanog analita, ali, ovisno o obliku testa, može uzrokovati i lažno smanjene vrijednosti analita (Slika 1Ab.)

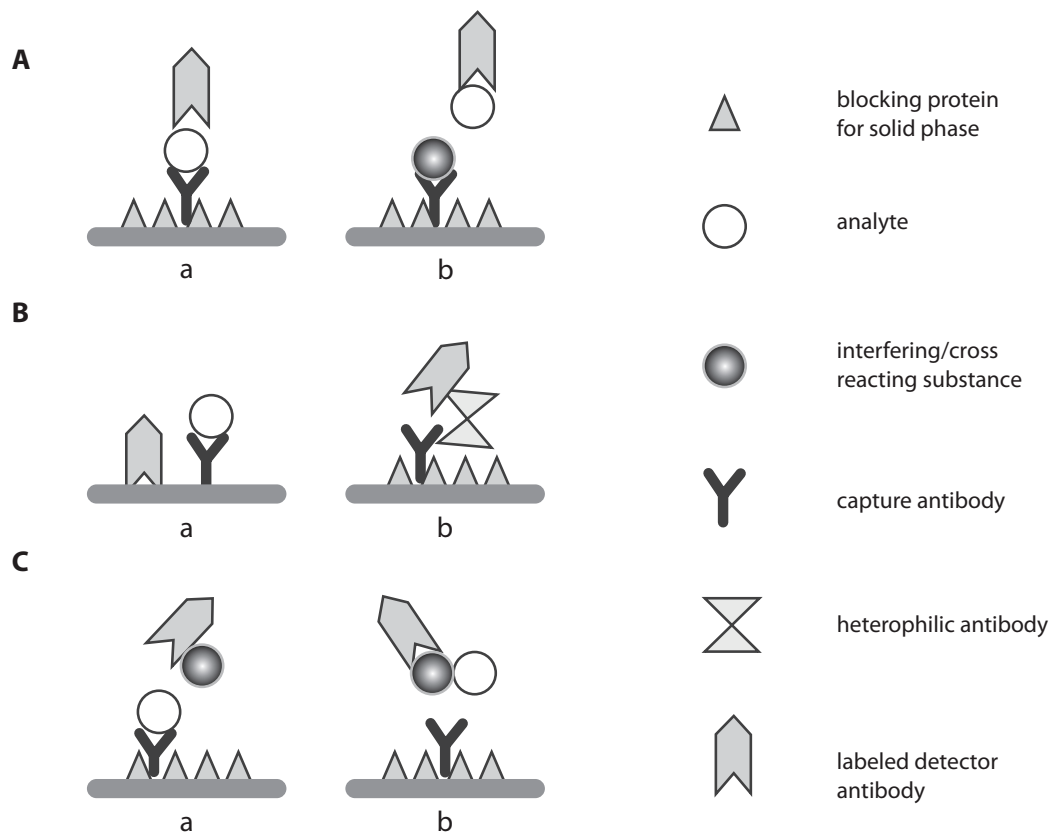
Križnu reaktivnost može prouzročiti metabolit ili prekursor analita, primjerice konjugirani metaboliti kortizola pri određivanju kortizola u mokraći (38), ili istodobna primjena lijekova slične strukture (triklički antidepresivi) (19). Poznat je problem križne reaktivnosti kod određivanja vitamina D ( $1,25\text{-[OH]}_2\text{D}_3$ ) zbog moguće pozitivne interferencije  $25\text{-OH D}_3$  (34). I u području alergologije poznate su interferencije (40) pri određivanju specifičnih IgE prema alergenima kravljeg mlijeka (41), alergenima grinja (42), plodovima mora (43), peluda i lateksa (44), epitela životinja (45), alergena otrova opnokrilaca (46). Opisana je također lažno povećana koncentracija IgE na alergene peluda biljaka, zbog prisustva IgE prema ugljikohidratnim determinantama monoglikozilirane alergenske molekule (47,48). U tom slučaju osoba, unatoč povećanoj koncentraciji IgE, nema simptoma alergijske bolesti. Razlog tome je taj, što IgE antitijela prema ugljikohidratnim determi-

genous and exogenous antibody-structured substances; 3) the hook effect; and 4) the matrix effect.

### Cross-reactivity with endogenous and exogenous non antibody-structured substances

Cross-reactivity is the most common interference in immunoassays, but mostly in competitive ones. It is non-specific influence of substances in a sample that structurally resembles analyte (carries similar or the same epitopes like analyte) and competes for binding site on antibody (34). The interference grade caused by cross-reactivity depends on three factors: antibody specificity, method and sample preparation (35). The most common examples can be seen during determination of hormone concentration, drugs, allergene-specific IgE. Hormones TSH (thyroid-stimulating hormone), LH (luteinizing hormone) and hCG (human chorionic gonadotropin) carry analogue  $\alpha$ -chain, and  $\beta$ -chain determines the specificity of the respective hormone – therefore, it is necessary to choose an assay which would be able to recognise different epitopes by using specific antibodies (36). The second example are steroid hormones, which have identical cyclopentanoperhydrophenanthrene structure (37,38). Furthermore, there is prostate specific antigen (PSA) which exists in several forms (total, PSA; free, fPSA; precursor, proPSA and newly also early prostate cancer antigen, EPCA) and causes cross-reactivity and insufficient accuracy (39). Cross-reactivity causes falsely elevated concentration of analyte, but depending on the test method falsely low values can also occur (Figure 1 Ab).

Cross-reactivity can be caused by metabolite or analyte precursor, e.g. conjugated cortisol metabolites by determining the urine cortisol (38) or simultaneous application of medications with similar molecular structure (tricyclic antidepressants) (19). The problem of cross-reactivity by vitamin D ( $1,25\text{-[OH]}_2\text{D}_3$ ) determination due to possible positive interference of  $25\text{-OH D}_3$  (34) is well known. In the area of allergology one can also find interferences (40) by determination of allergene-specific IgE to cow milk (41), mite allergens (42), seafood (43), pollen and latex (44), animal epithelium (45), hymenoptera sting venom allergens (46). Falsely increased concentration of IgE to pollen allergens due to presence of IgE to carbohydrate determinant of the monoglycosylated allergenic molecule have also been reported (47,48). In this case a person despite increased IgE concentration shows no symptoms of an allergic disease. The reason for that is that IgE antibodies to carbohydrate determinants do not affect histamine release from basophilic granulocytes or mast cells. Cross-reactivity usually causes positive interference, but in some assays negative interference is also possible. So, for example in digoxin-determinating assay oleandrin



**SLIKA 1.** Različite interferencije u imunokemijskim analizama: Aa - analiza bez interferencije; Ab - križna reaktivnost interferenta s veznim antitijelom, rezultira lažno negativnim rezultatom; B - pozitivna interferencija: a - nespecifično vezanje detektorskog antitijela na krutu podlogu koja nije obložena blokirajućim agensom; b - premoštavanje s heterofilnim antitijelima odnosno HAMA; C - negativna interferencija; Ca - promjena steričke konformacije zbog vezanja interferirajućeg proteina uz Fc fragment detektorskog antitijela; Cb - prikrivanje epitopa na analitu površine uzorka

**FIGURE 1.** Different interferences in immunoassays: Aa - assay without any interference; Ab - cross-reactivity of an interfering substance with capture antibody, resulting with false negative result; B - positive interference: Ba - unspecific binding of labelled detector (tracer) antibody to a not blocked solid phase; Bb - "bridge" binding by heterophilic antibodies or HAMA, respectively; C - negative interference: Ca - change of sterical conformation after binding of interfering protein to Fc fragment of detector antibody Cb - masking of the epitope on analyte surface by a protein of the sample.

nantama ne utječu na oslobađanje histamina iz bazofilnih granulocita odnosno mastocita.

Križna reaktivnost obično uzrokuje pozitivnu interferenciju, ali je u nekim testovima moguća i negativna interferencija. Tako npr. oleandrin (srčani glikozid sličan digoksinu) u testu određivanja digoksina može interferirati na različite načine (49). Pri smanjenim koncentracijama digoksina, oleandrin može imati pozitivnu interferenciju, a pri povećanim koncentracijama digoksina negativnu. U eri transplantacije organa osobito je važno znati da imunokemijske metode za određivanje koncentracije imunosupresivnog lijeka ciklosporina A daju značajno veću koncentraciju nego referentna metoda HPLC (49). Križna reaktivnost opisana je i u metodama za probiranje na

(digoxin-like cardiac glycoside) can interfere in different ways (49). By decreased digoxin concentrations, oleandrin can have positive interference and by increased concentrations negative interference. In the time of organ transplantations it is important to know that immunoassays for determination of cyclosporine A concentration, an immunosuppressive drug give significantly higher concentration than referent HPLC method (49). Cross-reactivity has also been reported in methods for drug misuse screening (50, 51). Faster dissociation of an interfering substance than of an analyte during washing or separating free from captured analyte during analysis can be the cause for falsely low concentration of an analyte (52).

zlouporabu lijekova (50,51). Uzrok lažno negativnim interferencijama može biti brža disocijacija interferenta nego analita pri ispiranju ili odvajanju slobodnog od vezanog analita tijekom analize (52).

U kompetitivnim metodama određivanja malih molekula (lijekovi), oba antitijela, primarno (vezno) i obilježeno (detektorsko), vežu se istodobno uz analit. Križnu je reaktivnost teško predvidjeti, stoga moramo biti svjesni njenog postojanja, pratiti stručno-znanstvenu literaturu i odabirati specifičnije metode.

### Križna reaktivnost s endogenim i egzogenim supstancama koje imaju strukturu antitijela

Na imunokemijsku reakciju mogu utjecati antitijela prisutna u biološkom uzorku bolesnika ili antitijela reagensa (13,53). Biološki uzorak može sadržavati egzogena i endogena antitijela. Endogena antitijela prisutna su u oko 40% osoba (14), osobito onih koje su dobivale imunoterapiju s monoklonalnim antitijelima (54). U skupinu egzogenih antitijela ubrajaju se imunološki lijekovi. Iz te se skupine najviše opisuje interferencija nakon intravenske primjene Fab fragmenta antidigoksinških antitijela (Fab fragment usmjeren prema antigenskoj determinanti digoksina; dobiva se iz antidigoksinških antitijela proizvedenih u ovci) (55,56). Mehanizam interferencije Fab fragmenta podrazumijeva različiti afinitet i specifičnost primarnih antitijela u pojedinim testovima. Opisana je interferencija ginsenga (imunoreaktivna tvar slična digoksinu) (57).

Dvije su vrste endogenih antitijela u serumu pacijenta. To su heterofilna antitijela (prirodna antitijela i autoantitijela) (58,59) i anti-animarna antitijela (engl. *human anti-animal antibodies*, HAAAs) (23). Iako se endogena antitijela razlikuju prema nekim svojstvima (60), interferiraju prema jednakom mehanizmu u saturacijskim analizama - stvaraju komplekse istodobno i s primarnim i obilježenim antitijelima reagensa te ih premoštavaju (Slika 1.). Heterofilna antitijela su multispecifična antitijela sintetizirana prema slabo definiranim antigenima. Humana anti-animarna antitijela su antitijela velike avidnosti, a sintetiziraju se prema dobro definiranim antigenima (16).

Interferencija heterofilnih antitijela većinom ima za posljedicu lažno povećane (Slika 1B.) rezultate (58,60-64), iako su opisani i lažno smanjeni rezultati (31,55,65-67) u slučajevima kad interferirajuće antitijelo stvara kompleks samo s jednim antitijelom iz reagensa. Pozitivna interferencija heterofilnih antitijela u sendvič metodama nastaje zbog toga što heterofilna antitijela premoštavaju primarno i obilježeno antitijelo (68). Negativna interferencija nastaje zbog vezanja heterofilnih antitijela izravno na primarno antitijelo, što onemogućuje vezanje analita. U nekim imunokemijskim metodama (ELISA, luminometrijske metode) reagensi sadrže životinjske proteine (goveđi albumin i kazein) koji služe za blokiranje reaktivnih

In competitive immunoassays of small molecules (drugs), both, the capture (binding) and the tracer antibody (labeled detector), bind simultaneously to the analyte. Cross-reactivity is hard to predict, therefore, we must be aware of its existence, be up to date with scientific literature and choose more specific methods.

### Cross-reactivity with endogenous and exogenous antibody-structured substances

Immunoreaction can be influenced by antibodies present in biological sample of a patient or antibodies from the reagent (13,53). Biological sample can contain exogenous and endogenous antibodies. Endogenous antibodies are presented in about 40% of patients (14), especially in those who were on immunotherapy with monoclonal antibodies (54). Immunological drugs belong to the group of exogenous antibodies. From this group the most commonly reported interference is the one upon intravenously applied Fab fragment from antidigoxin antibodies (Fab fragment is directed to antigen determinant of digoxin; Fab fragment comes from antidigoxin antibody produced in sheep) (55,56). Interference mechanism of the Fab fragment has different affinity and specificity of capture antibodies in some assays. It has also been reported about interference of ginseng (digoxin-like immunoreactive component) (57).

There are two types of endogenous antibodies in patients' serum. Heterophilic antibodies (natural antibodies and autoantibodies) (58,59) and anti-animal antibodies (human anti-animal antibodies; HAAAs) (23). Although endogenous antibodies differ in some characteristic (60) they interfere according to identical mechanism in saturating (sandwich) analyses - they simultaneously create complexes with capture and tracer antibodies of the reagent - they "bridge" them (Figure 1). Heterophilic antibodies are multi-specific antibodies synthesised to very poorly defined antigens. Human anti-animal antibodies are antibodies of high avidity and are synthesised to well defined antigens (16).

Heterophilic antibody interference has falsely elevated (Figure 1B) results as a consequence (58,60-64), although falsely low values (31,55,65-67) have been reported in cases when the interfering antibody creates a complex with only one antibody from the reagent. Positive heterophilic antibody interference in sandwich capture assays occurs because heterophilic antibodies "bridge" the capture and the tracer antibody (68). Negative interference occurs due to binding of heterophilic antibodies directly to the capture antibody what disables binding of analytes. In some assays (ELISA, luminometric methods) reagents contain animal proteins (bovine albumin and casein) which block reactive sites on microtiter plates or polystyrenic micro substances. However, their interference ability can

mjesta na mikrotitarskim pločicama ili polistirenskim mikročesticama. Međutim, dvojaka je mogućnost njihove interferencije: mogu uzrokovati lažno povećane rezultate, ali mogu izazvati i povećani pozadinski signal (engl. *background*), ako se heterofilna antitijela izravno vežu na njih (68). Heterofilna antitijela interferiraju i pri određivanju citokina metodom ELISA (69), čija je koncentracija u serumu vrlo mala (66,70). Kako bi se izbjegao utjecaj heterofilnih antitijela pri određivanju koncentracije citokina, uzorku se dodaje ne-imuni animalni serum (68). Moguće su interferencije i u bolesnika s monoklinalnim gamapatijama (69,71) i u bolesnika s prisutnim autoantitijelima (31,72).

Najpoznatija anti-animalna antitijela su humana anti-mišja antitijela (engl. *human anti-mouse antibodies*, HAMA) (14,53,73,74). Mišja monoklonalna antitijela sve se više primjenjuju intravenski u dijagnostičke ili terapijske svrhe u onkologiji (75), alergologiji (76), autoimunim bolestima (77), a neki pacijenti prema njima sintetiziraju HAMA. HAMA mogu interferirati s mišjim monoklinalnim antitijelima, ako su ona sastavni dio reagensa. Oko 10% pacijenata ima heterofilna antitijela (12), a oko 40% osoba koje su intravenski primile mišja monoklonalna antitijela sintetizirat će HAMA. Pojavnost antianimalnih antitijela veća je u bolesnika s manjkom IgA (39% ih ima anti-kozja, a 18% ih ima HAMA), nego osoba s normalnom koncentracijom IgA (22). Neke osobe mogu sintetizirati anti-animalna antitijela nakon što su bile izložene ostalim životinjskim antigenima, primjerice u cjepivima dobivenim u zečevima ili kokošima, antizmijskim otrovom dobivenim u konju, profesionalnim kontaktima s kućnim ljubimcima i ostalim životinjama epitela životinja (45).

Reagensi za imunometrijske analize obično sadrže serum ili neki drugi blokirajući agens, koji bi trebao smanjiti nespecifičnu interferenciju (20,53,78-81). Ako se očekuje interferencija, korisno je imati dodatni blokirajući agens kojim bi se tretirali uzorci (78). Ne postoji univerzalni blokirajući agens za sve analite i za sve metode, nego se mora primijeniti onaj agens koji je nakon validacije pokazao da najbolje reducira utjecaj heterofilnih antitijela za određeni analit (53).

### Prozonski učinak (engl. *hook effect*)

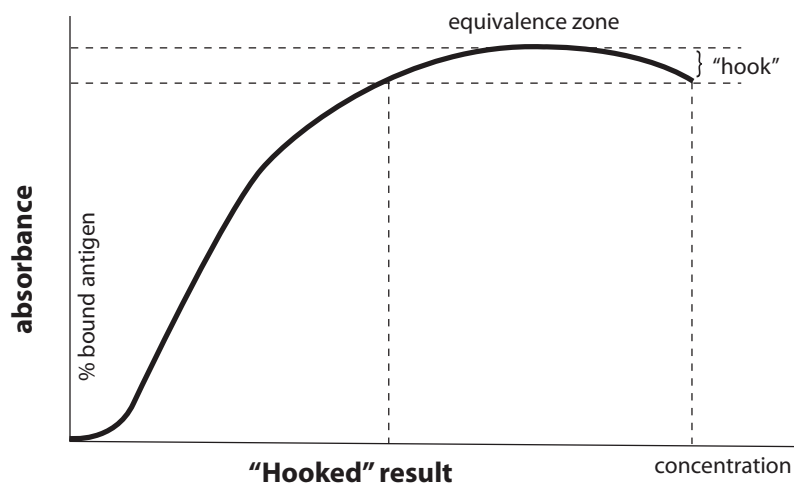
Prozonski učinak, u literaturi poznat i kao *hook* (engl. *hook*, savijen, poput udice) efekt, temelji se na krivulji zasićenja antitijela antigenom (Slika 2.). Primarno, prozonski učinak ovisi o koncentraciji analita (34,82-85). Podrazumijeva stanje njegova izrazitog suviška, koji zasiti sva vezna mjesta na antitijelu (86-89). Učinak nastaje uglavnom (ali ne isključivo), u metodama kod kojih se sve tri sastavnice (antigen, antitijelo, biljeg) inkubiraju istodobno (engl. *single step assay*) (90). Prozonski učinak ne postoji kod kompetitivnih imunokemijskih analiza. To znači da u reakciji ostaje višak analita koji nije ušao u sastav kompleksa

go in two directions: they can cause falsely increased results, but also induce increased background signal if the heterophilic antibodies directly bind to them (68). Heterophilic antibodies interfere also during cytokine determination by ELISA (69) whose serum concentration is very low (66,70). Heterophilic antibody interference in cytokine determination can be reduced by adding normal non-immune animal serum to the sample (68). Interferences in patients with monoclonal gammopathies (69,71) are also possible, as well as interferences in patients with present autoantibodies (31,72).

The most common anti-animal antibodies are human anti-mouse antibodies (HAMA) (14,53,73,74). Mouse monoclonal antibodies are increasingly applied intravenously in diagnostic or therapeutic purposes in oncology (75), allergology (76), autoimmune diseases (77), and some patients synthesise HAMA to them. HAMA can interfere with mouse monoclonal antibodies if they are a component of the reagent. Around 10% of patients carry heterophilic antibodies (12) and around 40% patients who intravenously received mouse monoclonal antibodies will synthesise HAMA. Prevalence of anti-animal antibodies is higher in patients with IgA deficiency (39% carry anti-goat antibodies and 18% carry HAMA) than in patients with normal IgA concentration (22). Some patients can synthesise anti-animal antibodies after exposure to other animal antigens, e.g. in vaccine produced in rabbits or chicken, in anti-snake venom produced in the horse, in professional exposure to pets and other animals (45). Reagents for saturating analyses usually contain serum or some other blocking agent that should diminish the non-specific interference (20,53,78-81). If interference is expected, it is useful to have an additional blocking agent to treat the samples (78). There is no universal blocking agent for all analytes and all methods. The agent that after validation showed as the best for reducing the effect of heterophilic antibodies for certain analyte must be applied (53).

### The hook effect

The hook effect is based on the saturation curve of antibody with antigen (Figure 2). Primarily, the hook effect depends on analyte concentration (34,82-85). It implies the presence of huge excess of analyte which saturates all binding sites on antibody (86-89). The effect occurs mostly (but not exclusively) in assays where all three components (antigen, antibody and marker) incubate simultaneously (single step assay) (90). The hook effect does not occur in competitive immunoassays. That means that in reaction there is a surplus on analytes that did not penetrate to analyte-antibody complex compound. This results in falsely decreased value of the measured analyte which could even lie in the reference interval. The value of



**SLIKA 2.** Prozonski učinak - Izrazito povećana količina analita nadmašuje vezni kapacitet primarnog antitijela. To rezultira neodgovarajuće slabim signalom koji uzrokuje pogrešno smanjen ili normalan rezultat ("hooked" result) u pacijenta kod kojeg postoji izrazito povećana koncentracija analita u serumu.

**FIGURE 2.** The hook effect - An excessive amount of analyte overwhelms the binding capacity of the capture antibody. This results in an inappropriately low signal that causes erroneous low or normal result ("hooked" result) for a patient with an excessively elevated serum analyte concentration.

analit-antitijelo. Posljedica jest lažno smanjena vrijednosti ispitivanog analita, koja može biti čak unutar referentnih intervala. Dobije se apsorpcija u post-zoni (silazna strana krivulje) čija je vrijednost jednaka vrijednosti apsorpcije u pre-/pro-zoni (uzlazna strana krivulje). U tom slučaju, reakcijska krivulja ima zvonolik oblik (engl. *bell-shaped curve*), odnosno savijena je poput udice (engl. *hook*) (34). Neki automatski analizatori imaju sustav za prepoznavanje suviška analita uz istodobno razrjeđivanje uzorka. Većina automatskih analizatora za područje kliničke kemije samo upozorava na nelinearnu reakciju, što je upozorenje da je uzorak potrebno razrijediti. Proizvođači reagensa za imunoturbidimetrijska određivanja smanjili su prozonski učinak uvođenjem lateks čestica kao nosača na kojima se odvija reakcija između analita (antigena) i antitijela. Kod kompetitivnih metoda prozonski je učinak otklonjen postupkom ispiranja (suvremeni automatski analizatori imaju programirano ispiranje) nakon reakcije analita s primarnim antitijelom i dodavanja obilježeng antitijela (86-90). Proizvođači reagensa smanjuju prozonski učinak povećanjem količine primarnog i obilježeng antitijela odnosno smanjenjem količine uzorka potrebnog za analizu. Prozonski učinak česta je pojava u svakodnevnom radu u kliničkim laboratorijima i nikako se ne smije zanemariti. Postoji kod onih analita koji se u serumu mogu naći u izrazito širokom rasponu koncentracija, kao što su primjerice C-reaktivni protein (stostruko povećanje), antistreptolizinska antitijela (deseterostruko povećanje), hormoni,

absorbance in the post-zone (down-side of the curve) is identical with the absorbance value in the pre-/pro-zone (up-side of the curve). In this case, the reaction curve is bell-shaped (bell-shaped curve) or hooked (34). Some automated analysers have a system for recognizing excess of an analyte while the sample is being simultaneously diluting. The most automated analysers used in clinical chemistry only warn about non-linear reaction what is sufficient to see if the sample needs to be diluted. Manufacturers of reagents for immuno-turbidimetric determinations have reduced the hook effect by introducing latex particles as carriers on which the reaction between analyte (antigen) and antibody takes place. In competitive assays the hook effect was eliminated by introducing a wash step (this wash step is programmed in current automated analysers) upon reaction of analyte with the capture antibody and addition of the tracer antibody (86-90). Reagents manufacturers reduce the hook effect by increasing the quantity of the capture and the tracer antibody and by reducing the quantity of samples required for the analysis.

The hook effect is common phenomenon in everyday work of a clinical laboratory and on no account should be neglected. It exists by analytes present in serum in extremely wide range of concentrations like C-reactive protein (100-fold increase), antistaphylococcal antibodies (10-fold increase), hormones (at 6-fold concentration increase) (hCG, IgE (>1000-fold), ferritin (100-fold increase), tu-



npr. hCG (kod šesterostrukog povećanja koncentracije), IgE (>1000 puta), feritin (stostruko povećanje), tumorski biljezi (osobito CA 19-9, PSA) (34). Na određivanje tumorskih biljega uglavnom utječe moguće veliko povećanje koncentracije (>10.000 puta), koje postoji u bolesnika s izrazitim tumorskim rastom. Može se pojaviti kod prve obrade bolesnika. Granična vrijednost, kod koje se gubi linearnost turbidimetrijskih metoda, pomaknuta je prema većim vrijednostima uvođenjem lateks čestica kao nosača antitijela. Ako proizvođač reagensa nije naznačio graničnu koncentraciju analita iznad koje se pojavljuje prozonski učinak, medicinski biokemičar bi to morao ispitati, te unijeti podatak u priručnik o kvaliteti rada laboratorija. Mogućnost postojanja prozonskog učinka otkriva se određivanjem uzorka s izrazito velikom koncentracijom u nerazrijeđenom uzorku i u razrijeđenjima, 1:10 i 1:100 (34,91). Ako se u razrijeđenim uzorcima dobije veći rezultat nego u nerazrijeđenom uzorku, radi se o prozonskom učinku. Potom slijedi određivanje granične koncentracije koja se može pouzdano odrediti. Uzorak se mora razrjeđivati sve dok se rezultati dvaju različitih razrjeđenja podudaraju (uzimajući u obzir faktor razrjeđenja). Ako se unaprijed očekuje izrazito povećana vrijednost ispitivanog analita koja će dati lažno smanjenu vrijednost, mogu se odmah pripremiti dva uzorka - nerazrijeđeni i razrijeđeni.

### Učinak matriksa

Uzorak seruma, odnosno plazme, složena je smjesa lipida, proteina, ugljikohidrata, soli i vode. Zbroj interferencija svih sastavnica u uzorku (osim analita), koje utječu na mjerenje ciljnog analita, poznat je pod nazivom „činak matriksa“ (92,93). Većina sastavnica seruma, koje uzrokuju tzv. učinak matriksa, imaju mali afinitet vezanja za analit ili antitijelo. Obično ta sastavnica maskira analit ili antitijelo, zbog čega izostaje reakcija vezanja analita s antitijelom. Osim svih endogenih elemenata (94), koji uzrokuju interindividualnu (25) i intra-individualnu varijabilnost (95-97) rezultata imunokemijskih analiza, pojam učinka matriksa mogao bi se proširiti i na egzogene sastavnice, koje se odnose na utjecaj antikoagulansa pri uzorkovanju plazme (98), odnosno na utjecaj aktivatora zgrušavanja krvi i separatora pri uzorkovanju seruma. Heparinska terapija bolesnika s akutnim infarktomiokarda utječe na rezultat određivanja troponina I (96). Heparin se zbog negativnog naboja polianiona, veže s kationima troponina (96). Rezultat toga mogu biti konformacijske promjene molekule troponina ili prikrivanje epitopa koji sudjeluju u imunokemijskoj reakciji s antitijelima reagensa. Osim toga, heparin se različitim afinitetom veže s pojedinim oblicima troponina, koji se mogu naći u krvi bolesnika u različitim fazama nakon infarkta miokarda (95). EDTA može djelovati na oslobađanje slobodnog cTnI iz kompleksa cTnI-troponin C

(especially CA 19-9, PSA) (34). Tumor marker determination is mostly influenced by possible huge concentration increase (>10,000 fold) that exists in patients with extreme tumor growth. It can occur at the initial laboratory workup of the patient. The cut-off value, where the linearity of turbidimetric methods is lost, is shifted towards higher values by introducing latex particles as antibody carriers. If the reagent manufacturer has not marked the cut-off analyte concentration above which the hook effect occurs, medical biochemist should investigate this and record this data into the manual on work quality of the laboratory. The possibility of the hook effect occurrence is discovered by determining sample with exceptionally high concentration in nondiluted form and in dilutions 1:10 i 1:100 (34,91). If in diluted samples higher values are measured than in nondiluted sample, we are talking about the hook effect. The reliable determination of cut-off concentration follows afterwards. The sample must be diluted until the results of two different dilutions match (taking into consideration the dilution factor). If extreme increased value of the measured analyte is expected, two samples could be prepared – the nondiluted and the diluted one.

### The matrix effect

Serum or plasma sample is a complex compound of lipids, proteins, carbohydrates, salt and water. The sum of interferences of all sample components (with exception of analytes), which affect the target analyte to be measured is known as “the matrix effect” (92,93). The most serum components that cause so called matrix effect have low affinity of binding to an analyte or antibody. This component disguises usually the analyte or the antibody causing the absence of the binding reaction of analyte to antibody.

Except of these endogenous elements (94), which cause inter- (25) and intra-individual variability (95-97) of results, the concept of the matrix effect could be widened to exogenous components that relate to the impact of anticoagulant during plasma sampling (98) or the impact of coagulation activator and separator during serum sampling. Heparin therapy in patients with acute myocardial infarction (AMI) affects the result of determination of troponin I concentration (96). Due to negative charge of polyanions heparin binds with cations of troponin (96). This can result either in conformational changes in troponin molecule or in directly covering epitopes involved in the immunoreaction with antibodies from the reagent. Besides, heparin binds with different affinity to some troponin forms present in patient's blood in different phases after myocardial infarction (95). EDTA can act upon release of free cTnI from calcium ion dependent cTnI-troponin C complex (96) what causes falsely decrease of values

ovisnog o ionima kalcija (96), što uzrokuje lažno smanjene vrijednosti u metodama koje sadrže antitijela usmjerena prema kompleksu troponina. Iako postoje preporuke da se srčani biljezi određuju u plazmi, osobito u hitnoj službi (99), za određivanje troponina uzorak izbora jest serum (može biti uzorkovan s gelom ili bez gela u epruveti) ili uzorak uzorkovan u epruvetu koja sadrži trombin (s ili bez gela) (95).

Gel koji služi kao separator seruma može adsorbirati analit, što može izazvati lažno smanjenu koncentraciju kod određivanja lijekova, primjerice antidepresiva, benzodiazepina (100). Pet do trideset % lijeka može se adsorbirati na gel, a ako uzorak stoji dulje vrijeme (24 h) adsorpcija može iznositi i do 40%. U slučaju da uzorak za analizu treba zamrznuti, serum se mora odvojiti u posebnu epruvetu (101). Činjenica da na tržištu postoji više vrsta epruveta s gelom različite kakvoće, potiče na oprez pri njihovom odabiru, odnosno ukazuje na potrebu njihove validacije pri određivanju pojedinih analita.

Uzrok varijabilnosti rezultata može biti i u matriksu kalibratora (102,103), odnosno kontrolnih uzoraka (20), jer nemaju istovjetan matriks kao biološki uzorak u kojem se neki analit određuje.

## Zaključak

Danas se imunokemijske analize ne primjenjuju samo u specijalističkim laboratorijima, nego i u općim medicinsko-biokemijskim laboratorijima (104), a pogotovo u privatnim laboratorijima. Imunometrijskim metodama određuju se hCG (105), hormoni štitnjače (20,106), srčani biljezi (29-31,107), tumorski biljezi (55,73,74,108,109) pa su kod tih analita opisivane i interferencije heterofilnih antitijela. Pojavnost interferencija manja je u analizama koje se primjenjuju dulje vrijeme (proizvođači reagensa i analizatora su ih nastojali otkloniti), nego u analizama koje su kratko u primjeni (110). Osobito treba obratiti pozornost na imunokemijske metode uz krevet bolesnika kod kojih je također opisana interferencija heterofilnih antitijela (111).

Poznavanje brojnih interferencija preduvjet je za njihovo prepoznavanje. Njihovim prepoznavanjem mogu se izbjeći moguće neželjene posljedice važne kako za bolesnika (pogreške u dijagnozi, liječenje i praćenje uspješnosti liječenja, nepotrebna terapija) tako i za zdravstveni sustav (nepotrebna dodatna istraživanja).

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in methods containing antibodies, directed to troponin complex. Despite the recommendations that heart markers should be determined in plasma, especially in emergency department (99), the sample of choice for troponin determination is serum, collected in tubes with or without gel or in thrombin tubes with or without gel (95).

Gel used as serum separator can adsorb analyte what can cause falsely low concentration of drugs prescribing, e.g. antidepressants, benzodiazepine (100). Five to thirty % of the drugs can be adsorbed on gel and if the sample is kept for longer time (24h) adsorption can rise up to 40%. In case the sample for analysis requires freezing, the serum must be placed into a separate test tube (101). There are several different types of test tubes with gel of different quality available on the market today. Therefore, one should be cautious in choosing them and the need for their validation by determination of some analytes is rising.

The cause for result variability may be in the matrix of the calibrator (102,103) or the control samples (17) due to the fact that their matrix is not identical with the biological sample in which some analyte is being determined.

## Conclusion

Today, immunoassays are not applied only in specialist laboratories but also in medical biochemical laboratories (104) and especially in private laboratories. Saturating methods are used for determination of hCG (105), thyroid hormones (20,106), cardiac marker (29-31,107), tumor markers (55,73,74,108,109) and in these analytes it has also been reported about interferences of heterophilic antibodies. Prevalence of interference is lower in analyses used for longer period of time (manufacturers of reagents and analysers tended to eliminate them) than in those which are in use for shorter period of time (110). Special attention must be paid to assays with bedside measurements where is also reported about interference of heterophilic antibodies (111).

Knowledge of numerous interferences is a prerequisite for their recognition which helps avoiding possible undesirable consequences important for the patient (diagnostic errors, treatment and monitoring of its efficacy, unnecessary therapy) and for the health care system as well (unnecessary additional researches).

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