Analitička procjena komercijalnog teksta za određivanje koncentracije P1NP

Analytical evaluation of commercial P1NP assay

Marko Marcius[1], Nada Vrkić[2], Biserka Getaldić-Švarc[2]

[1]Farmaceutsko-biokemijski fakultet Sveučilišta u Zagrebu, Zagreb

[1]School of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia

[2]Klinički zavod za kemiju, KB Sestre milosrdnice, Zagreb, Hrvatska

[2]Klinical Institute of Chemistry, Sestre milosrdnice University Hospital, Zagreb, Croatia

Sažetak

Cilj: Ispitati značajke testa ukupnog P1NP na biološkom materijalu u uvjetima kliničkog laboratorija, te rezultate procjene usporediti sa svojstvima testa koje je deklarirao proizvođač.

Metode: Analitička procjena testa P1NP provedena je prema protokolu Europskog odbora za kliničko-laboratorijske standarde (ECCLS). Analit amino terminalni propeptid tipa I prokolagena (ukupni P1NP) određen je elektrokemiluminescentnom imunometodom na automatskom analizatoru Elecsys 2010 (Roche Diagnostics, Mannheim, Njemačka).

Rezultati: Zadovoljavajuće rezultate dala su ispitivanja nepreciznosti u seriji kao i nepreciznosti iz dana u dan kroz 10 dana. Odstupanja od deklariranih vrijednosti kontrolnih uzoraka pokazala su zadovoljavajući stupanj točnosti, a nisu ni u jednom mjerenju prelazila granicu od navedene 1 SD. Ispitivanje linearnosti te procjena interferencije s hemoglobinom potvrdili su u potpunosti navode proizvođača. Štoviše, prisutnost hemolize nije pokazala utjecaj sve do koncentracije hemoglobina od 1,61 mmol/L, što je 32% veća koncentracija od one koju navodi proizvođač. Rezultati ispitivanja utjecaja lipemije i hiperbilirubinemije pokazali su nešto slabije rezultate u usporedbi s deklariranim graničnim vrijednostima. Potvrđena je interferencija bilirubina i triacilglicerola u koncentracijama većim od 610 µmol/L, odnosno 15,0 mmol/L, što je prosječno 30% niže od navoda proizvođača.

Zaključak: Test ukupni P1NP prilagođen je rutinskom radu u medicinsko-biokemijskom laboratoriju. Automatski analizator Elecsys 2010 (Roche Diagnostics, Mannheim, Njemačka) pruža selektivnost, brzinu, jednostavnost rukovanja, stabilnost kalibracija, te računalnu potporu u praćenju kontrolnih i bolesničkih uzoraka.

Ključne riječi: analitička procjena, amino terminalni propeptid tipa I prokolagena, P1NP, elektrokemiluminescentna imunometoda, koštani biljezi

Abstract

Aim: Evaluation of the characteristics of total P1NP assay performed on biological material in clinical laboratory conditions, and comparison of evaluation results with the assay properties declared by the manufacturer.

Methods: Analytical evaluation of P1NP assay was conducted according to the European Committee for Clinical Laboratory Standards (ECCLS) protocol. Amino-terminal propeptide of type I procollagen (P1NP) was determined by electrochemiluminescent immunoassay on an Elecsys 2010 automated analyzer (Roche Diagnostics, Mannheim, Germany).

Results: Satisfactory results were achieved for within-run and between-run imprecision during 10 days. Deviation from declared control sample values showed a satisfactory level of accuracy, not exceeding the limit of declared 1 SD on any measurement. Study results for linearity and assessment of hemoglobin interference entirely confirmed the manufacturer's declarations. Moreover, the presence of hemolysis showed no interference due to hemoglobin up to 1.61 mmol/L (32% higher than that stated by the manufacturer). Interference results for lipemia and hyperbilirubinemia (>610 µmol/L and 15.0 mmol/L, respectively) showed poorer values compared to those declared. Bilirubin and triacylglycerol interference was confirmed in concentrations that were on an average by 30% lower than those declared by the manufacturer.

Conclusion: Total P1NP test is suitable for routine use in medical laboratory. The Elecsys 2010 automated analyzer ensures rapidity, simple manipulation, calibration stability, control samples, and computer support for the analysis of control and patient samples.

Key words: analytical evaluation, amino terminal propeptide type I procollagen, P1NP, electrochemiluminescent immunoassay, bone markers

Pristiglo: 21. kolovoza 2006. *Received: August 21, 2006.*

Prihvaćeno: 16. listopada 2006. *Accepted: October 16, 2006.*

Uvod

Odabir i procjena metode važni su koraci u organizaciji kvalitetnog medicinsko-biokemijskog laboratorija. Prije same primjene nove metode nužna je njezina procjena u kliničkim uvjetima, bez obzira radi li se o ručnom ili automatskom izvođenju mjerenja, ili o reagensima pripravljenim u laboratoriju, ili o komercijalnom proizvodu. Za primjenu nove metode u laboratoriju ponajprije je potrebno izmjeriti analitičku nepreciznost i netočnost, a potom ispitati linearnost metode i utjecaj interferirajućih supstanca. Na laboratorijski analitički rezultat utječu prvenstveno slučajna i sustavna pogreška koje zajedno čine mjernu pogrešku. Stoga analitički rezultat predstavlja pravu vrijednost umanjenu odnosno uvećanu za veličinu mjerne pogreške (1).

Biokemijski biljezi koštane pregradnje

Biokemijski su biljezi koštane pregradnje više od tri desetljeća važni klinički pokazatelji u dijagnostici koštanih bolesti (Pagetova bolest, rahitis, osteomalacija, primarni i sekundarni hiperparatiroidizam, renalna osteodistrofija, osteoporoza). No, još uvijek kliničaru ne daju dovoljno osjetljive i specifične pretrage u koje bi se mogli posve pouzdati u dijagnostici i terapiji, a koje bi uz to mogle zamijeniti invazivnu i skupu tehniku biopsije kostiju zdjelice za histomorfometrijsku analizu (2,3).

Biljezi koštane pregradnje biološke su molekule koje se pojavljuju u cirkulaciji ili se izlučuju mokraćom odražavajući dinamiku koštanog metabolizma. Brzina pregradnje procjenjuje se mjerenjem serumskih aktivnosti enzima koji izravno sudjeluju u koštanom metabolizmu ili mjerenjem sadržaja kolagenih i nekolagenih proteinskih struktura koštanog matriksa u serumu i mokraći (4,5). Brojni su biljezi razvrstani u dvije temeljne grupacije: za procjenu koštane izgradnje (osteokalcin, alkalna fosfataza, N- i C-terminalni propeptidi prokolagena) i za procjenu razgradnje (tartarat-rezistentna kisela fosfataza, piridinolin, deoksipiridinolin, hidroksiprolin, kalcij, N- i C- terminalni telopeptidi). Svaki od biljega zahtijeva pažljivu procjenu u specifičnom kliničkom stanju budući da je njihova razina u krvi i mokraći izložena brojnim čimbenicima nevezanim za koštani metabolizam. Primjena metode neinvazivnog denzitometrijskog mjerenja gustoće kostiju (BMD), koja je otpočetka osigurala prihvatljivu preciznost i točnost, prihvaćena je među kliničarima, ali učinci terapije zbog sporog koštanog metabolizma tom se metodom mogu uočiti tek nakon godinu dana primjene terapije. Biokemijski su biljezi pokazali kliničku vrijednost upravo u bržoj procjeni uspješnosti terapije u usporedbi s denzitometrijskom metodom (3,6,7).

Posljednjih petnaestak godina znanstveni izazov u traganju za boljim razumijevanjem bazične biokemije i fiziologije kostiju, a time i metaboličnih koštanih bolesti, urodio je nizom osjetljivijih i specifičnijih biljega koštane preg-

Introduction

Method selection and evaluation are important steps in organizing high quality medical laboratory. Prior to its application, a new method should undergo evaluation in clinical setting, regardless of whether manual or automated measurements, in-house reagents, or a commercial product are in question.

The application of a new method in the laboratory requires previous measurement of analytical imprecision and inaccuracy followed by determination of the method linearity and effect of interfering substances. Laboratory analysis results are primarily influenced by random or systemic errors, which together make the measurement error. Therefore, an analysis result represents the real value decreased, or increased, for the value of the measurement error (1).

Biochemical markers of bone turnover

Biochemical markers of bone turnover have for more than three decades been important clinical indicators in the diagnosis of bone diseases (Paget's disease, rickets, osteomalacia, primary and secondary hyperparathyroidism, renal osteodystrophy, osteoporosis). They, however, still do not provide clinicians with sufficiently sensitive and specific data on which they could entirely rely in the diagnosis and therapy, and which could replace invasive and expensive biopsy technique for histomorphometric analysis of pelvic bones (2,3).

The markers of bone turnover are molecules that occur in blood and urine, and reflect the dynamics of bone metabolism. Bone turnover is assessed by measuring serum activities of the enzymes directly involved in bone metabolism, or by measuring the content of collagen fragments and noncollagen proteins of bone matrix in serum and urine (4,5). Numerous markers have been classified into two main groups: markers for evaluation of bone formation (osteocalcin, bone alkaline phosphatase, N- and C-terminal procollagen propeptides), and those for evaluation of bone degradation (tartarate-resistant acid phosphatase, pyridinoline, deoxypyridinoline, hydroxyproline, calcium, N- and C-terminal telopeptides of type I collagen). Each of the markers requires careful evaluation in a specific clinical condition, as their urine and blood concentrations also depend on numerous factors not related to bone metabolism. The application of non-invasive measurement of bone mineral density (BMD) as a „gold standard" has ensured acceptable precision and accuracy since its introduction and has been accepted among clinicians, yet therapeutic effects based on this method may be observed not earlier than at one/two years due to slow bone metabolism. Therefore, biochemical markers demonstrated their clinical value in the possibility of more rapid assessment of therapeutic success in comparison with densitometry (3,6,7).

During the past fifteen years, the scientific endeavors directed towards the search for better understanding of the basic bone biochemistry and physiology, and thereby also

radnje. Međutim, preciznost i točnost ručnih metoda pokazale su nezadovoljavajuću razinu. Međulaboratorijski koeficijenti varijacije čak do 71%, uz interindividualnu varijabilnost do 27% predstavljali su velik analitički problem. Stoga su do danas razvijene i dostupne mnoge automatizirane metode, a njihova je primjena preduvjet daljnjih istraživanja (3,8,9,10,11).

Ukupni P1NP

Ukupni N-terminalni propeptid tipa I prokolagena (ukupni P1NP) noviji je test koji je razvila tvrtka Roche Diagnostics za automatski analizator Elecsys (Roche Diagnostics, Njemačka) i koji je kao intaktni koštani biljeg izgradnje već pokazao brojne kliničke prednosti (12,13). Osobito se to odnosi na primjenu kod uremičnih bolesnika, jer je klirens P1NP posredovan receptorima u jetrenim endotelnim stanicama i potpuno je neovisan o bubrežnoj disfunkciji (14).

Na slici 1. prikazana je molekula prokolagena tipa I (prekursorski oblik kolagena) iz koje otkidanjem peptida specifičnim endopeptidazama s N- i C-terminalnog kraja nastaje kolagen tipa I, temeljni protein koji izgrađuje oko 90% koštanog matriksa. Slobodni propeptidi kolaju krvlju i svjedoče o živoj sintezi kolagena.

Ovaj rad imao je za cilj analitičku validaciju, reagensa za određivanje koncentracije P1NP.

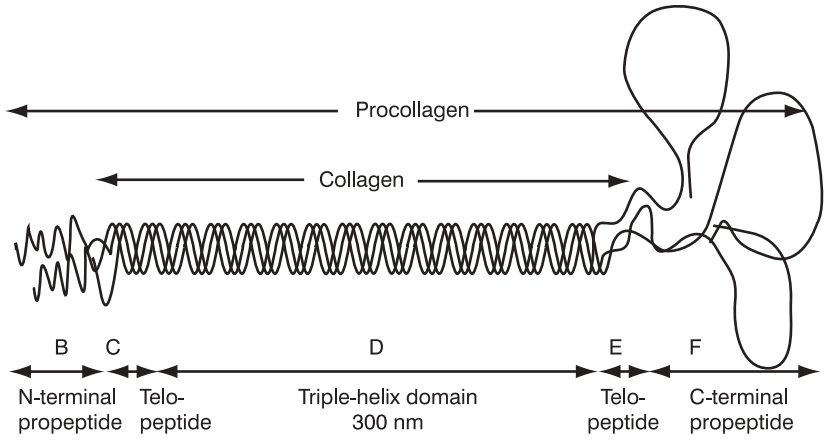
of metabolic bone diseases, led to the discovery of a series of sensitive and specific markers of bone turnover. Nevertheless, the precision and accuracy of the manual methods employed for the measurement of these markers turned out to be of unsatisfactory quality. Inter-laboratory coefficients of variation reaching as high as 71% and inter-individual variability of up to 27% were a considerable analytical issue. Therefore, many automated methods have currently been developed and become available, and their application is a prerequisite for further investigations (3,8,9,10,11).

Total P1NP

Total N-terminal propeptide of type I procollagen is a test recently developed for Elecsys immunochemistry analyzer (Roche Diagnostics, Germany). As a marker of intact bone formation, this test demonstrated numerous clinical advantages (12,13), mainly related to its application in uremic patients since P1NP clearance is mediated by receptors in hepatic endothelial cells and is completely independent of renal dysfunction (14).

Figure 1 shows a molecule of type I procollagen (a form of precursor collagen) which, after specific endopeptidases have detached peptides from the N- and C-terminal end, produces type I collagen, a basic protein that forms approximately 90% of bone matrix. Free propeptides circulate in blood and demonstrate collagen synthesis.

The aim of this study was analytical validation, of P1NP assay.



Slika 1. Shematski prikaz prokolagena tipa I s domenama koje predstavljaju biokemijskie biljege. Posebno je označena domena P1NP. (prema Prockop DJ, Kivinkko KI, Tuderman L, Guzman NA. The biosynthesis of collagen and its disorders. N Engl J Med 1979;301:12-23, 77-85.). B - Amino-terminalni propeptid tipa I prokolagena – P1NP; C - Amino-terminalni poprečno vezani telopeptid tipa I kolagena – INTP; D - Trostruka zavojnica; E - Karboksi-terminalni poprečno vezani telopeptid tipa I kolagena – ICTP; F - Karboksi-terminalni propeptid tipa I prokolagena – PICP; C + D + E – Kolagen

Figure 1. Graphic presentation of type I procollagen with domains representing biochemical markers. P1NP domain is specifically designated (according to Prockop DJ, Kivinkko KI, Tuderman L, Guzman NA. The biosynthesis of collagen and its disorders. N Engl J Med 1979;301:12-23, 77-85). B - Amino-terminal propeptide of type I procollagen propeptide – P1NP; C - Amino-terminal cross-linking telopeptide of type I collagen – INTP; D - triple helix; E - Carboxy-terminal cross-linking telopeptide of type I collagen – ICTP; F - Carboxy-terminal propeptide of type I procollagen– PICP; C + D + E – Collagen

Materijali i metode

Reagensi

Koncentracija ukupnog P1NP mjerena je originalnim test reagensom Total P1NP (03141071 Roche Diagnostics, Mannheim, Njemačka) na analitičkom sustavu Elecsys iste tvrtke, a kalibracija u dvjema točkama provedena je također izvornim setom kalibratora (Total P1NP CalSet 03141080190, Roche Diagnostics, Mannheim, Njemačka). Metoda je standardizirana prema referentnom materijalu definiranom preciznom odvagom nativnog P1NP u matrici humanog seruma slobodnog od analita (23). Reagensi su čuvani na temperaturi od 2-8 °C.

Uzorci ispitanika s koncentracijama P1NP iznad gornje granice analitičkog područja koje smo rabili za određivanje linearnosti metode razrjeđivani su izvornim diluentom (Elecsys Diluent Universal, 03183971122) koji sadrži protein matriks i konzervanse.

Za ispitivanje interferencija dodavane su u uzorke seruma slijedeće interferirajuće supstance pripravljene kao otopine: Fresenius Kabi Intralipid ™ 20% (Fresenius Kabi, AB, Uppsala, Švedska); bilirubin u prahu (Kemika, Zagreb) i pripravak hemoglobina iz hemolizata 5 mL slučajno uzete krvi s EDTA.

Uzorci

Ispitivanja nepreciznosti i netočnosti metode provedena su s kontrolnim uzorcima PreciControl Bone (163883, Roche Diagnostics, Mannheim, Njemačka) na tri koncentracijske razine: niska PC1 (73,5±5,87 μg/L), srednja PC2 (508,0±35,7 μg/L) i visoka PC3 (978,0±68,3 μg/L) te na humanim serumima iz ostatne krvi bolesnika. Za ispitivanje linearnosti metode i interferencija rabljeni su humani serumi. Uzorci krvi uzimani su tijekom 15 dana između 7 i 10 sati ujutro u epruvete s podtlakom (Beckton Dickinson) bez antikoagulansa. Serumi su odvajani nakon centrifugiranja (10 min na 3500 okretaja/min) te su priređena dva *pool* seruma (*pool* I. i *pool* II.) za testiranje nepreciznosti i zasebna tri *pool* seruma za ispitivanje interferencija. Uzorke seruma s visokim koncentracijama P1NP, odabrane među kroničnim bubrežnim bolesnicima na trajnoj hemodijalizi, upotrijebili smo za priređivanje još dva *pool* seruma (*pool* III. i *pool* IV.) kojima smo ispitivali linearnost metode.

Određivanje koncentracije ukupnog P1NP

Koncentracija P1NP mjerena je elektrokemiluminescentnom (ECLIA) sendvič tehnikom s dvama monoklonskim protutijelima. Serumski, kontrolni uzorak ili kalibrator (20 μL) inkubira se sa specifičnim monoklonskim protutijelom na P1NP koje je obilježeno biotinom. Nakon dodatka mikročestica obilježenih streptavidinom i monoklonskih protutijela na P1NP obilježenih rutenij kompleksom (Tris(2,2`-bipiridil)rutenij(II)-kompleks ($Ru(byp)_3^{2+}$)) stvara se i fiksira sendvič kompleks na čvrstoj fazi (mikročesticama) zahvaljujući reakciji biotina i streptavidina. P1NP iz uzorka nalazi

Materials and methods

Reagents

Total P1NP concentration was measured using an original test reagent Total P1NP (03141071, Roche Diagnostics, Mannheim, Germany) on an Elecsys analytical system manufactured by the same company, with two-point calibration also performed using an original calibrator set (Total P1NP CalSet 03141080190; Roche Diagnostics, Mannheim, Germany). This method has been standardized against reference standards precisely defined by weighing native P1NP into an analyte-free human serum matrix (23). The reagents were kept at a temperature of 2-8 °C.

Subjects' samples with P1NP concentrations above the upper detection limit that were used to determine the method linearity were diluted using an original diluent (Elecsys Diluent Universal, 03183971122) containing protein matrix and preservatives.

To examine interference, the following interfering substances were prepared as solutions and added to serum samples: 20% Fresenius Kabi Intralipid™ (Fresenius Kabi, AB, Uppsala, Sweden), bilirubin powder (Kemika, Zagreb, Croatia), and hemoglobin preparation from 5 mL hemolysate of randomly collected blood with EDTA.

Samples

Analyses of imprecision and inaccuracy of the method were performed using PreciControl Bone control samples (163883, Roche Diagnostics, Mannheim, Germany) at three concentration levels: low PC1 (73.5±5.87 μg/L), medium PC2 (508.0±35.7 μg/L) and high PC3 (978.0±68.3 μg/L), and on residual samples of human sera. To determine the method linearity and interference human sera were used. Blood samples were collected during 15 days between 7 and 10 a.m. into Vacutainer test-tubes (Becton Dickinson) without anticoagulant. The sera were separated following centrifugation (10 min at 3,500 rpm), two serum pools (pool I and pool II) were prepared to test imprecision and three serum pools to examine interference. Serum samples with high P1NP concentrations, selected among patients with chronic renal insufficiency on continuous dialysis, were used to prepare another two serum pools (pool III and pool IV) that were used to investigate the method linearity.

Total P1NP concentration measurement

P1NP concentration was measured by electrochemiluminescence (ECLIA) sandwich immunoassay with two monoclonal antibodies. A serum, control sample or calibrator (20 μL) was incubated with P1NP-specific biotin-labeled monoclonal antibodies. After the addition of streptavidin-labeled microparticles and a monoclonal P1NP-specific antibody labeled with ruthenium complex (Tris(2,2`-bipyridil)ruthenium(II)-complex (Ru(byp)32+)), a sandwich complex was formed and bound to the solid

se u sendviču dvaju protutijela. Potom se reakcijska smjesa aspirira u mjernu stanicu gdje se mikročestice vežu za površinu elektrode, a nevezane se čestice ispiru pomoću otopine ProCell. Napon na elektrodi uzrokuje prijenos elektrona te kemiluminescentnu emisiju rutenijskog kompleksa čiji se signal mjeri luminometrijski. Svi navedeni postupci, kao i provjera valjanosti kalibracije izvode se automatski na sustavu Elecsys 2010, a analiza traje 18 minuta.

Kalibracija

Svaki set reagensa za određivanje ukupnog P1NP ima barkod-zapis koji sadrži informacije o kalibraciji iz prisutne serije reagensa, odnosno zapis o glavnoj tzv. "*master*" krivulji koju priređuje tvrtka Roche kalibrirajući cijelo analitičko područje s 10 kalibratora. Kalibraciju u dvije točke, preciznije podudarnost s glavnom kalibracijskom krivuljom, proveli smo s pripadajućom serijom niskog i visokog kalibratora.

Analitička procjena

Analitička procjena provedena je prema preporukama Europskog odbora za kliničko-laboratorijske standarde (ECCLS) (15).

Ispitivanje nepreciznosti

Nepreciznost u seriji određena je s po 30 uzastopnih mjerenja za dva *pool* seruma srednje i visoke koncentracije P1NP i izražena je kao koeficijent varijacije, KV(%). Nepreciznost iz dana u dan određena je mjerenjem koncentracije kontrolnih seruma PC 1, PC 2 i PC 3 u duplikatu kroz 10 dana. U izračunu nepreciznosti rabila se je srednja vrijednost dnevnih mjerenja, a rezultati su izraženi kao KV(%).

Ispitivanje netočnosti

Netočnost mjerenja prikazana je kao postotak odstupanja R(%) srednje izmjerene vrijednosti ($\bar{x}$) od srednje deklarirane vrijednosti (S) primijenjenih kontrolnih uzoraka. Srednja izmjerena vrijednost rabljena u izračunu dobivena je ispitivanjem nepreciznosti iz dana u dan kroz 10 dana. Odstupanje, tzv. "*bias*", od očekivane vrijednosti izračunato je prema matematičkom izrazu $(\bar{x}\text{-}S)/S \times 100\%$.

Ispitivanje linearnosti

Linearnost metode ispitana je mjerenjem koncentracije P1NP u nizu razrjeđenja dvaju *pool* seruma s koncentracijama P1NP blizu i iznad gornje granične vrijednosti od proizvođača deklarirane linearnosti. U *pool* serumu I. linearnost je ispitana nizom od 7 razrjeđenja (do 1:200), a u pool serumu II. nizom od 5 razrjeđenja (do 1:150). Proizvođač navodi područje linearnosti od 5 do 1200 µg/L.

Ispitivanje interferencija

Ispitivane su interferencije hiperbilirubinemije, lipemije i hemolize. U *pool* serum dodavane su različite količine interferirajućih supstanca do najviše koncentracije bilirubina od 1948,80 µmol/ L, hemoglobina do 2,32 mmol/L i

phase (microparticles) due to the biotin and streptavidin interaction. The reaction mixture was then aspirated into the measuring cell where microparticles bound to the electrode surface, while unbound particles were rinsed using ProCell solution. The voltage applied to the electrode caused electron transfer and chemiluminescent emission of the ruthenium complex whose signal was determined luminometrically. All the above procedures and testing of calibration validity were performed automatically on the Elecsys 2010 system; the analysis took 18 minutes.

Calibration

Each reagent set for total P1NP determination has a barcode label with information on calibration of the particular reagent lot, and information on the master calibration curve prepared by Roche by calibration of the entire analytical range using 10 calibrators. Two-point calibration, or more accurately the compliance with the master calibration curve, was performed with the provided series of low and high calibrators.

Analytical evaluation

Analytical evaluation was performed according to the European Committee on Clinical Laboratory Standards (ECCLS) recommendations (15).

Analysis of imprecision

Within-run imprecision was established by 30 subsequent measurements of two serum pools with medium and high P1NP concentrations, and expressed as coefficient of variation, CV (%). Between-run imprecision was determined by measuring the concentration of control sera PC1, PC2 and PC3 in triplicate over 10 days. The mean of daily measurements was used to calculate imprecision, and results were expressed as CV (%).

Analysis of inaccuracy

Inaccuracy of measurements was evaluated by measuring the values of analytes in control samples during 10 days. It is expressed as a percentage of bias R (%) of the determined mean value ($\bar{x}$) from the declared mean value (S). As a measure of inaccuracy we used the percentage R(%) which is expressed as a bias from the declared value. It is calculated in relation to the mean measured value $\bar{x}$ according to the mathematical formula: $(\bar{x}\text{-}S)/S \times 100\%$.

Analysis of linearity

The linearity of the method was examined by measuring P1NP concentration in a series of dilutions of two serum pools with P1NP concentrations near and above the upper linearity limit as declared by the manufacturer. In serum pool I, linearity was analyzed through a series of seven dilutions (up to 1:200), and in serum pool II through a series of 5 dilutions (maximum 1:150). The manufacturer declared a linearity range of 5-1,200 µg/L.

triacilglicerola do 20,0 mmol/L, a zatim su u duplikatu određene koncentracije P1NP. Priprema interferirajućih otopina i dilucijskog niza provedena je prema preciznom protokolu Haeckela (16).

Statistička analiza

Statističko je računanje provedeno programom Excel (Microsoft Office for Windows XP Professional) rukovodeći se statističkim načelima Westgarda i suradnika za deskriptivnu statistiku i linearnu regresiju (17-19). Rabili smo slijedeće statističke veličine za procjenu testa: srednja vrijednost ($\bar{x}$), standardna devijacija (SD), koeficijent varijacije (KV), postotak odstupanja (R), koeficijent korelacije (r) i parametri jednadžbe pravca (a i b) za odnose vrijednosti prema metodi linearne regresije.

Rezultati

Nepreciznost

Koeficijenti varijacije KV u seriji od po 30 uzoraka za dva *pool* seruma *pool* I. i *pool* II. (koncentracija P1NP 46,7±1,00 μg/L i 642,6±14,82 μg/L) iznosili su 2,14% i 2,31%. Nepreciznost iz dana u dan ispitana je na kontrolnim uzorcima PC1, PC2, PC3 (vrijednosti P1NP 66,6±0,95 μg/L; 474,3±8,12 μg/L i 893,3±18,27 μg/L) izražena kao KV iznosila je 1,44%, 1,71% i 2,04%. Tablica 1 prikazuje rezultate nepreciznosti metode u seriji, dok Tablica 2 prikazuje rezultate nepreciznosti metode iz dana u dan.

Analysis of interference

Analysis of interferences included hyperbilirubinemia, lipemia and hemolysis. Different quantities of interfering substances were added to serum pools but not exceeding the concentration of 1,948.80 μmol/L for bilirubin, 2.32 mmol/L for hemoglobin, and 20.0 mmol/L for triacylglycerol. Then P1NP concentrations were determined in duplicate. The preparation of solutions and dilution sequence was conducted according to the precise protocol by Haeckel (16).

Statistical analysis

Statistical calculation was performed using Excel application (Microsoft Office for Windows XP Professional) according to statistical principles by Westgard et al. for descriptive statistics and linear regression (17-19). The statistical values used for test evaluation were as follows: mean ($\bar{x}$), standard deviation (SD), coefficient of variation (CV), percentage of bias (R), coefficient of correlation (r), and linear regression parameters (a and b) for value relations according to the linear regression method.

Results

Imprecision

Resultes are presented in Table 1. and 2. Coefficients of variation (CV) obtained in batch mode of 30 sample runs for two serum pools, pool I and pool II (P1NP concentrations 46.7±1.00 μg/L and 642.6±14.82 μg/L) were

Tablica 1. Nepreciznost u seriji (n=30). Pool I. – normalne koncentracije P1NP (μg/L), pool II. – visoke koncentracije P1NP (μg/L)

Table 1. Within-run imprecision (n=30). Pool I – normal P1NP concentrations (μg/L); pool II – high P1NP concentrations (μg/L)

N=30	Pool I	Pool II
$\bar{x}$ [μg/L]	46.7	642.6
SD [μg/L]	1.00	14.82
CV [%]	2.14	2.31

Tablica 2. Nepreciznost iz dana u dan (n=20). Kontrolni serumi na tri koncentracijske razine (PC1, PC2, PC3)

Table 2. Between-run imprecision (n=20). Control sera at three concentration levels (PC1, PC2, PC3)

N=20	Control sera PC 1	Control sera PC 2	Control sera PC 3
$\bar{x}$ [μg/L]	66.6	474.3	893.3
SD [μg/L]	0.95	8.12	18.27
CV [%]	1.44	1.71	2.04

Netočnost

Rezultati su predočeni u tablici 3 koja prikazuje standardnu devijaciju (SD), koeficijent varijacije (KV) te veličinu odstupanja R od deklariranih vrijednosti P1NP u kontrolnim uzorcima izraženu u % i ona iznosi 9,28% za PC1, 6,19% za PC2 i 8,53% za PC3.

2.14% and 2.31%, respectively. Between-run imprecision was determined on control samples PC1, PC2, and PC3 (P1NP concentrations 66.6±0.95 μg/L, 474.3±8.12 μg/L and 893.3±18.27 μg/L, respectively) and expressed as CV (1.44%, 1.71% and 2.04%, respectively).

Inaccuracy

Results are presented in Table 3. The bias R from P1NP declared values in control sera was expressed in percentage. It was 9.28%, 6.19% and 8.53% for PC1, PC2 and PC3, respectively.

Tablica 3. Netočnost određivanja P1NP primjenom kontrolnih uzoraka u tri koncentracijske razine (n=20; PC1, PC2, PC3 – kontrolni uzorci niske, srednje i visoke koncentracijske razine)

Table 3. Inaccuracy of P1NP determination at three concentration levels (n=20; PC1, PC2, PC3 – commercial control sera with low, medium and high concentration level, respectively)

N=20	Control sera PC 1	Control sera PC 2	Control sera PC 3
Measured values P1NP ($\bar{x}$) [μg/L]	66.6	474.3	893.3
Expected values P1NP [μg/L]	73.5	508.0	978.0
SD [μg/L]	0.95	8.12	18.27
Bias R [%]	–9.28	–6.19	+ 8.53

Linearnost

Linearnost smo ispitali dilucijskim testom na dva *pool* seruma za dvije koncentracijske razine: na polovici mjernog područja (544,8 μg/L) i iznad mjernog područja (>1 200 μg/L). Najmanji postotak odstupanja od očekivanog rezultata (7,2% i 2,4%) pokazalo je razrjeđenje 1:2. Rezultati dilucijskih testova prikazani su na Tablici 4 te na slikama 2 i 3.

Interferencije

Mjereći koncentracije P1NP u serumima kojima su dodavane rastuće koncentracije interferirajućih supstanca ispitivali smo utjecaj na točnost određivanja. Predmet našega ispitivanja bile su najčešće interferencije u humanim serumima uzrokovane hiperbilirubinemijom, hiperlipemijom i hemolizom. Rezultati prikazani na slikama 4, 5 i 6 i tablicama 5, 6 i 7 pokazuju negativnu devijaciju rezultata zbog njihova utjecaja. Interferencija je ustanovljena uz onu koncentraciju supstance koja uzrokuje odstupanje rezultata >10% (opće prihvaćenoj granici za analite kojima nije poznata biološka varijabilnost).
Sniženu vrijednost izmjerenog P1NP za više od 10% našli smo u uzorcima s bilirubinom ≥610 μmol/L, triacilglicerolom ≥15,0 mmol/L i hemoglobinom ≥1,61 mmol/L.

Linearity

Linearity was examined using dilution test on two serum pools for two concentration levels: medium value of the measuring range (544.8 mg/L) and values above the measuring range (>1200 μg/L). The lowest per cent of deviation from the expected result (7.2% and 2.4%) was recorded at 1:2 dilution. Dilution test results are presented in Table 4 and Figures 2 and 3.

Interferences

Measurement accuracy was examined by measuring P1NP concentrations in the sera supplemented with increasing concentrations of interfering substances. The subject of our examination were the most frequent interferences in human sera that are caused by hyperbilirubinemia, hyperlipemia and hemolysis. The data presented in Figures 5, 6 and 7 show negative deviation of results due to the effect of these interferences. An interference was established if the substance concentration caused >10% bias in results (this is a widely recognized limit for analytes with unknown biological variability). P1NP value decreased by >10% was measured in samples with bilirubin ≥610 μmol/L, triacylglycerol ≥15.0 mmol/L and hemoglobin ≥1.61 mmol/L.

Tablica 4. Rezultati dilucijskog testa na dvije koncentracijske razine za ispitivanje linearnosti P1NP testa

Table 4. Dilution test results at two concentration levels to assess P1NP assay linearity

		Sera P1NP, mg/L		
Pool serum	**Dilution**	**Expected**	**Measured**	**Recovery, %**
III	1:1 (undiluted)	544.8	544.8	100.0
	1:2	272.4	252.8	92.8
	1:3	181.6	158.6	87.3
	1:5	108.9	76.1	69.9
	1:10	54.4	28.9	53.1
	1:20	27.2	6.3	23.2
	1:50	10.8	< 5	/
IV	1:1 (undiluted)	/	> 1,200	/
	1:2	1197	1197	100.0
	1:4	598.5	584.3	97.6
	1:10	239.4	170.8	71.3
	1:20	119.7	64.8	54.1
	1:50	47.9	18.4	38.4
	1:100	23.9	7.5	32.1
	1:200	11.9	< 5	/

Tablica 5. Utjecaj bilirubina na točnost određivanja P1NP

Table 5. Effect of bilirubin on the P1NP assay accuracy

bilirubin [µmol/L]	P1NP [µg/L]	Bias [%]
9,3	226,3	± 0,00
101,5	228,8	1,10
232,8	234,7	3,71
386,6	230,4	1,81
504,0	228,0	0,75
747,6	187,9	-16,97
1948,8	107,9	-52,32

Tablica 6. Utjecaj triacilglicerola na točnost određivanja P1NP

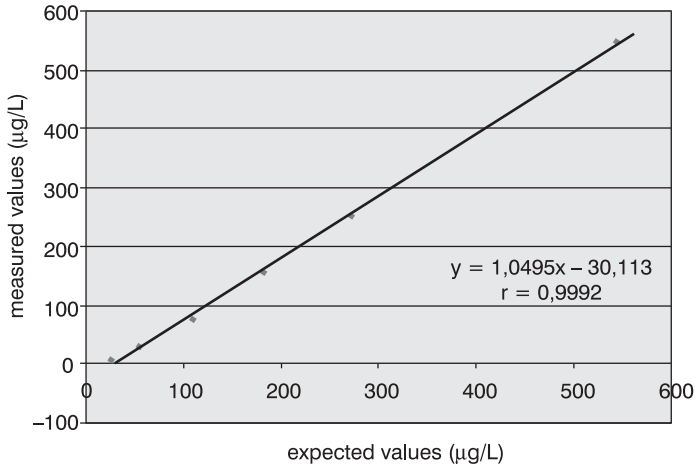
Table 6. Effect of triacylglycerol on the P1NP assay accuracy

Triacylglycerol [mmol/L]	P1NP[µg/L]	Bias [%]
1,21	28,7	± 0,00
1,83	28,0	-2,44
2,41	27,9	-2,79
3,63	27,4	-4,53
6,15	27,0	-5,92
9,80	26,5	-7,67
12,52	26,0	-,41
18,75	25,1	-12,55

Tablica 7. Utjecaj hemoglobina na točnost određivanja P1NP

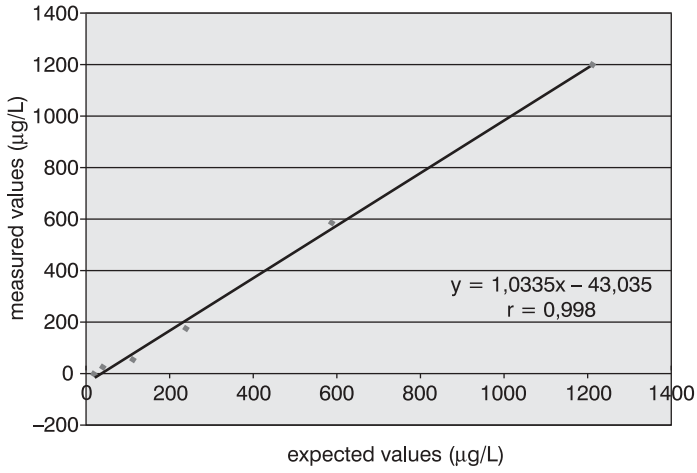
Table 7. Effect of hemoglobin on the P1NP assay accuracy

haemoglobin [mmol/L]	P1NP[µmol/L]	Bias [%]
0	28,9	± 0,00
0,28	28,5	-1,38
0,58	27,8	-3,81
0,71	27,5	-4,84
1,61	27,0	-6,57
2,32	21,9	-24,22



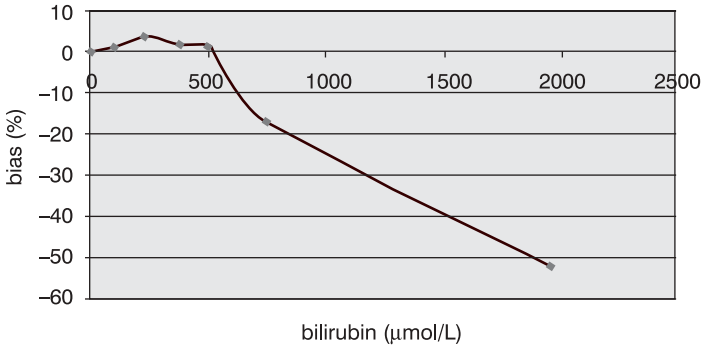
Slika 2. Dilucijski test za P1NP test na uzorku seruma koncentracije 544,8 µg/mL.

Figure 2. Dilution test for P1NP assay in pool serum with P1NP value of 544.8 µg/mL.



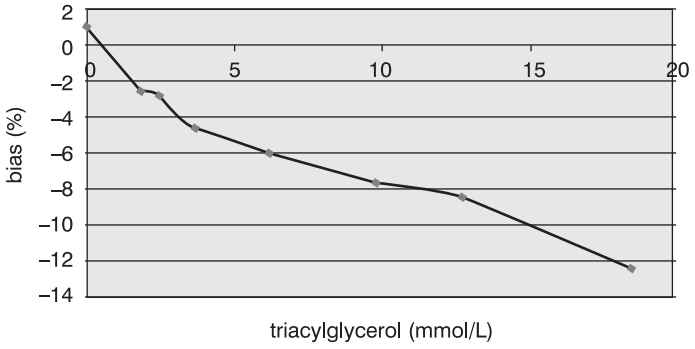
Slika 3. Dilucijski test za P1NP na uzorku seruma koncentracije >1 200 µg/mL.

Figure 3. Dilution test for P1NP assay in pool serum with P1NP value of >1,200 µg/mL



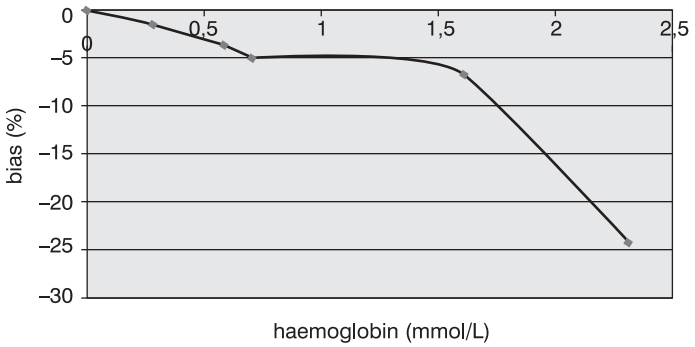
Slika 4. Utjecaj bilirubina na točnost određivanja P1NP. (P1NP=226,3 μg/mL)

Figure 4. Effect of bilirubin on the P1NP assay accuracy. (P1NP=226.3 μg/mL)



Slika 5. Utjecaj triacilglicerola na točnost određivanja P1NP. (P1NP=28,7 μg/mL)

Figure 5. Effect of triacylglycerol on the P1NP assay accuracy. (P1NP=28.7 μg/mL)



Slika 6. Utjecaj hemoglobina na točnost određivanja P1NP. (P1NP=28,9 μg/mL)

Figure 6. Effect of hemoglobin on the P1NP assay accuracy. (P1NP=28.9 μg/mL)

Rasprava

Metoda denzitometrijskog mjerenja mineralne gustoće kostiju (BMD) kao neinvazivna i lako dostupna metoda otvorila je nove mogućnosti istraživanja metaboličnih koštanih bolesti. Potaknulo je to i biokemijska i farmaceutska istraživanja te su danas realna očekivanja da će biokemijski biljezi koštanog metabolizma osigurati neke kliničke prednosti pred BMD. Potvrdu takvih promišljanja nalazimo u činjenici da je, unatoč dobroj preciznosti metode BMD, potrebno godinu dana (za sporu koštanu pregradnju i dvije godine) da bismo uz 95% pouzdanosti mogli uočiti promjene u koštanoj gustoći. Taj se problem ne uočava u velikim kliničkim studijama gdje se značajne koštane promjene skupine zapažaju nakon 6 mjeseci (2,3,19), nego u individualnoj kontroli terapije (20,21). Specifičnost i osjetljivost koštanih biljega vrijedne su značajke kojima će se možda prebroditi navedene manjkavosti BMD.

P1NP je novi biljeg koji tehnikom ECLIA s dva monoklonska protutijela omogućuje mjerenje ukupnog (intaktnog) propeptida, nasuprot radioimunokemijskoj komercijalnoj metodi PINP koja s jednim protutijelom detektira i razgrađene dijelove propeptida. Stoga su opravdana očekivanja kliničara kako će primjenom ove metode moći provjeravati i modulirati terapiju u vremenu kraćem od godine dana (12,13).

Naše je istraživanje provedeno kako bismo ispitali analitičke osobine metode koja je prirađena za automatski analizator zatvorenog sustava. Jednostavna je za provođenje svih analitičkih postupaka, s informatičkim prednostima u standardizaciji laboratorijskog postupka i povezivanju s laboratoriskim informacijskim sustavom.

Budući da ne poznajemo ukupnu intraindividualnu i biološku varijabilnost koncentracije P1NP nismo mogli postaviti kriterije kojima bismo mogli vjerodostojnije procijeniti nepreciznost i netočnost. Prihvaćeni kriteriji za analite s poznatim podacima o varijabilnosti su slijedeći: dozvoljene granice za nepreciznost iznose manje od polovice intraindividualne varijacije ($KV<½KV_{intra}$), a dozvoljene granice za netočnost iznose manje od jedne četvrtine ukupne biološke varijacije (*bias* % $<¼ KV_B$) (22). Proizvođač navodi i literaturom potkrepljuje da cirkadijalni i sezonski ritam koncentracije P1NP ima varijacije oko 6%. Načinom uzorkovanja ovu smo varijabilnost sveli na najmanju moguću mjeru, jer su sve krvi uzimane u isto doba dana (između 7 i 10 sati ujutro) i u isto doba godine (unutar 15 dana).

Za neke druge koštane biljege (primjerice, C-terminalni telopeptid) postoje podatci kako intraindividualna varijabilnost iznosi 9–10% (22). Proizvođač navodi za P1NP nepreciznost od 1,8% do 2,9%, a naši su se rezultati potvrdili s KV od 1,4% do 2,3%. Iznenadili su bolji rezultati KV za nepreciznost iz dana u dan (1,4%; 1,7%; 2,0%) negoli za nepreciznost u seriji (2,1% i 2,3%), što možemo objasniti homogenijim i bolje priređenim kontrolnim uzorcima nego što su *pool* serumi priređeni u laboratoriju.

Discussion

As a noninvasive and easily available method, the measurement of bone mineral density (BMD) has created new possibilities for investigating metabolic bone diseases. It has also encouraged biochemical and pharmaceutical studies, and current realistic expectations are that biochemical markers of bone metabolism are to offer some clinical advantages over BMD. Such reflections may be supported by the fact that, despite good precision of the BMD method, one year is necessary (for slow bone remodeling even two years) for changes in bone density to be observed with 95% reliability. This problem is not noticed in large clinical studies where significant bone changes in a group are observed after six months (2,3,19), but rather in individual therapy monitoring (20,21). The specificity and sensitivity of bone markers are valuable characteristics that may help overcome the foregoing BMD disadvantages.

The novel P1NP assay allows for the measurement of total (intact) propeptide, a bone formation marker, through the ECLIA (Roche Diagnostics) technique involving two monoclonal antibodies, unlike radioimmunochemical commercial PINP method that detects components with only one antibody. Therefore, clinicians' expectations that the use of this method is to allow for therapy verification and modification in a time span shorter than half a year are quite warranted (12,13).

Our study was conducted with the aim to examine analytical properties of the method prepared for an automated closed-system analyzer. The method ensures simple performance of all analytical procedures, and offers advantages in standardization of laboratory procedures and integration into the laboratory information system.

Having been unaware of the entire intra-individual and biological variability of P1NP concentration, we were not able to establish the criteria for more reliable assessment of imprecision and inaccuracy. The accepted criteria for analytes with known variability data are: allowed imprecision limits amounting to less than a half of intra-individual variation ($CV<½ CV_{intra}$), and the allowed inaccuracy limits amounting to less than one fourth of the total biological variation (bias % $<¼ CV_B$) (22). The manufacturer states, supporting it with references, that circadian and seasonal changes of P1NP concentration show approximately 6% variation. We reduced this variability to the minimum by the sampling method employed, as all blood samples were collected at the same time of the day (between 7 and 10 a.m.) and at the same time of year (within 15 days). The data regarding some other bone markers (e.g., C-terminal telopeptide) revealed 9%-10% intra-individual variability (22). The manufacturer declares 1.8%-2.9% imprecision for P1NP, and our results were consistent with the CV ranging from 1.4% to 2.3%. Results for the CV for between-run imprecision were more surprising (1.4%, 1.7% and 2.0%) than for within-run imprecision (2.1% and 2.3%),

Ispitivanje netočnosti na kontrolnim uzorcima nije ni na jednoj koncentracijskoj razini dalo veće odstupanje od 10% u odnosu na deklariranu srednju vrijednost proizvođača. Takav rezultat u potpunosti zadovoljava. Štoviše, nijedno pojedinačno mjerenje kroz 10 dana nije izišlo iz granica ±1 SD, što je kriterij prihvatljivosti u provođenju dnevne kontrole.

Ispitivanje linearnosti dilucijskim testom potvrdilo je linearni odnos u analitičkom području (r>0.9) s proporcionalnim odnosom očekivanih i izmjerenih koncentracija P1NP (a=1,049 i 1,033). Ujedno je potvrđena preporuka proizvođača da se vrijednosti P1NP iznad gornje granice analitičkog područja razrjeđuju samo u omjeru 1:2. Kako se vidi iz jednadžbe pravca na slikama 2. i 3., svako slijedeće razrjeđenje udaljavalo nas je od početne koncentracije, što je izraženo velikom konstantnom pogreškom (b=-30,11 i -43,04) i objašnjava zbog čega se u sve većim razrjeđenjima pogreška uvećava. Nadalje, u uputama proizvođača osobito se napominje da se u razrjeđenim uzorcima bolesnika s bubrežnom insuficijencijom zapaža nelinearni odnos. *Pool* serume s visokim sadržajem P1NP prikupili smo upravo od uzoraka nefroloških bolesnika koji su na trajnoj hemodijalizi.

Koncentracije interferirajućih supstanca koje uzrokuju odstupanja od točnosti za više od 10% nisu se u potpunosti slagale s navodima proizvođača. Utjecaj hemolize zapažen je kao negativna interferencija uz koncentraciju hemoglobina od 1,61 mmol/L, što je za 32% veća koncentracija od deklariranih 1,1 mmol/L. Lipemični i ikterični serumi također su pokazali negativne interferencije uz sadržaj triacilglicerola ≥15,0 mmol/L i bilirubina ≥610,0 9 μmol/L, što je 35% odnosno 46% niža koncentracija od navoda proizvođača (22,8 mmol/L i 1112,9 μmol/L).

I u zaključku, nova metoda ECLIA za P1NP pokazuje jako dobre analitičke značajke za rutinsku laboratorijsku uporabu. Jednostavan je i pouzdan test s čijim rezultatima interferiraju samo ekstremne hiperbilirubinemije, hiperlipidemije i hemolize. Instrumentacija i računalna oprema prilagođena je testu i sigurno vodi korisnika kroz analitički postupak. Rezultati su u skladu s relevantnim literaturnim podacima (24,25). Sveukupnost analitičkih svojstava testa P1NP tvrtke Roche Diagnostics ostvaruje preduvjete za daljnja klinička ispitivanja koja će procijeniti i njegovu kliničku primjenjivost.

which may be attributed to the more homogeneous and better prepared control sera in comparison to laboratory-prepared serum pools.

Examination of inaccuracy in control samples yielded values that did not exceed 10% bias at any concentration level in relation to the mean value declared by the manufacturer. Such a result was completely satisfactory. Moreover, none of the measurements performed during 10 days exceeded the limits of ±1SD, which is a criterion of acceptability in daily control performance.

Examination of linearity by the dilution test confirmed linear relationship in the analytical range (r>0.9) with proportional relation between the expected and measured P1NP concentrations (a=1.049 and 1.033). Also, the manufacturer's recommendation was confirmed that the P1NP values exceeding the upper measuring range limit are to be diluted only at a 1:2 ratio. As evident from the linear regression in Figures 2 and 3, each additional dilution took us further from the initial concentration, as expressed by a substantial constant error (b=-30.11 and -43.04), explaining why the error increased with higher dilution. Furthermore, the manufacturer's instructions particularly specify that non-linear relation is observed in diluted samples from patients with renal insufficiency. Indeed, serum pools with a high P1NP content included samples collected from patients on continuous hemodialysis.

The concentrations of interfering substances that caused more than 10% bias did not entirely comply with the manufacturer's declarations. The effect of hemolysis was observed as a negative interference at a hemoglobin concentration exceeding 1.61 mmol/L, which was a concentration higher by 32% than the declared 1.1 mmol/L. Lipemic and icteric sera also demonstrated negative interferences in case of the content of triacylglycerol ≥15.0 mmol/L and bilirubin ≥610.0 μmol/L, which was by 35% and 46% lower than the concentration declared by the manufacturer (22.8 mmol/L and 1112 μmol/L, respectively).

In conclusion, the new ECLIA method for P1NP determination demonstrated very good analytical characteristics for routine laboratory use. It is a simple and reliable assay with interferences recorded only in case of extreme hyperbilirubinemia, hyperlipidemia and hemolysis. Our results were consistent with literature data (24,25). The analyzer and computer equipment are test adjusted and guide the user safely through the analytical procedure. The comprehensive analytical properties of the P1NP assay by the Roche Diagnostics provide a prerequisite for clinical trials that are to assess its clinical applicability.

Adresa za dopisivanje:
Marko Marcius
Prepuštovečki odvojak 4
10360 Sesvete
e-pošta: *marko_marcius@yahoo.co.uk*
tel. 098 686 206

Corresponding author:
Marko Marcius
Prepuštovečki odvojak 4
10360 Sesvete, Croatia
e-mail: *marko_marcius@yahoo.co.uk*
phone. +385 98 686 206

Literatura / References

1. *Hammer-Plećaš A, Čvorišćec D, Stavljenić-Rukavina A. Analitička kontrola u medicinsko biokemijskom laboratoriju. Biochemia Medica 1995;5:37-45.*
2. *Vrkić N, Krpan D, Primorac D. Medicinsko laboratorijska dijagnostika bolesti koštanog sustava. U: Topić E, Primorac D, Janković S, ur. Medicinskobiokemijska dijagnostika u kliničkoj praksi. Zagreb: Medicinska naklada; 2004.*
3. *Kleerekoper M. Biochemical markers of bone turnover: why theory, research, and clinical practice are still in conflict? Clin Chem 2001;47:1347-9.*
4. *Marcus R. Biochemical assessment of bone resorption and formation. Bone 1996;18 (Suppl):15S-16S.*
5. *Delmas PD. Biochemical markers of bone turnover. Acta Orthop Scand 1995;66:176-82.*
6. *Ginty F, Cavadini C, Michaud P-A, et al. Effect usual nutrient intake and vitamin D status on markers of bone turnover in Swiss adolescents. Eur J Clin Nutr 2004;58:1257-65.*
7. *Okabe R, Nakatsube K, Inaba M, et al. Clinical evaluation of the Elecsys β-CrossLaps serum assay, a new assay for degradation product of type I collagen C-telopeptides. Clin Chem 2001;47:1410-4.*
8. *Seibel MJ, Lang M, Geilenkeuser WJ. Interlaboratory variation of biochemical markers of bone turnover. Clin Chem 2001;47:1443-50.*
9. *Garnero P, Borel O, Delmas PD. Evaluation of a fully automated serum assay for C-terminal cross-linking telopeptide of type I collagen in osteoporosis. Clin Chem 2001;47:694-702.*
10. *Schmidt-Gayk H, Spanuth E, Kötting J, et al. Performance evaluation of automated assay for β-CrossLaps, N-MID-osteocalcin and intact parathyroid hormone (BIOROSE Multicenter Study). Clin Chem Lab Med 2004;42:90-5.*
11. *Seibel MJ, Woitge HW, Farahmand I, Oberwittler H, Ziegler R. Automated and manual assays for urinary crosslinks of collagen: which assay to use? Exp Clin Endocrinol Diabetes 1998;106:143-8.*
12. *Reid IR, Davidson JS, Wattie D, et al. Comparative response of bone turnover markers to bisphosphonate therapy in Paget's disease of bone. Bone 2004;35:224-30.*
13. *Thurairaje R, Iles RK, Jefferson K, McFarlane JP, Persad RA. Serum amino-terminal propeptide of type I procollagen (P1NP) in prostate cancer: a potential predictor of bone metastases and prognosticator for disease progression and survival. Urol Int 2006;76:67-71.*
14. *Ueda M, Inaba M, Okuno S, et al. Clinical usefulness of the serum N-terminal propeptide of type I collagen as a marker of bone formation in hemodialysis patients. Am J Kidney Dis 2002;40:802-9.*
15. *European Committee for Clinical Laboratory Standards. Guidelines for evaluation of analyzers in clinical chemistry. Beuth Verlag Berlin, Köln ECCLS Document, 1986;3:1-32.*
16. *Haeckel R. Evaluation methods in laboratory medicine. Berlin: VCH; 1993.*
17. *Westgard JO, Carey RN, Wold S. Criteria for judging precision and accuracy in method development and evaluation. Clin Chem 1974;20:825-33.*
18. *Westgard JO, de Vos DJ, Hunt MR, et al. Method evaluation. Houston: American Society for Medical Technology, 1978.*
19. *Yow L, Sayre JW. Statistical concepts in the interpretation of serial bone densitometry. Invest Radiol 1994;29:928-32.*
20. *Johnston CC Jr, Bjarnason NH, Cohen FJ, Shaha A, et al. Long-term effect of raloxifene on bone mineral density, bone turnover, and serum lipid levels in early postmenopausal women: three-year data from 2 double-blind, randomized, placebo-conrolled trials. Arch Intern Med 2000;160:3444-50.*
21. *Wallach S, Choen S, Reid DM, Hughes RA, et al. Effect of riserdronate treatment of bone density and vertebral fracture in patients on corticosteroid therapy. Calcif Tissue Int 2000;67:277-85.*
22. *Fraser CG, Peterson H, Ricos C, Haeckel R. Proposed quality specifications for imprecision and inaccuracy of analytical system for clinical chemistry. Eur J Clin Chem Clin Biochem 1992;30:311-7.*
23. *Data on file at Roche Diagnostics.*
24. *Sebba AI, Bonnick SL, Kagan R, Thompson DE, Skalky CS, et al. Response to therapy with once-weekly alendronate 70 mg compared to once-weekly risedronate 35 mg in the treatment of posmenopausal osteoporosis. Curr Med Res Opin 2004;20:2031-41.*
25. *Jung K, Lein M, Stephan C, Von Hosslin K, Semjonow A, et al. Comparison of 10 serum bone turnover markers in prostate carcinoma patients with bone metastatic spread: diagnostic and prognostic implications. Int J Cancer 2004;111:783-91.*