Ocjena HPLC sustava Primus Ultra^2 za mjerenje koncentracije HbA_2 i probir na β-talasemiju

Evaluation of the Primus Ultra^2 HPLC system for HbA_2 measurement and β-thalassemia screening

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Sažetak

Uvod: Primus Ultra^2 je analizator na kojem se metodom tekućinske kromatografije visoke djelotvornosti (engl. *high-performance liquid chromatography*, HPLC) pomoću ionske izmjene obavlja kvantitativna i kvalitativna analiza frakcija hemoglobina: u 4 minute moguće je odrediti točnu koncentraciju HbF, HbA_2, HA_0 i detektirati druge vrste hemoglobina. Cilj istraživanja je ocjena analitičkog učinka analizatora Primus Ultra^2 u rutinskoj procjeni sadržaja HbA_2, najvažnijeg testa za identifikaciju nosioca β-talasemije.

Materijali i metode: Provedena su istraživanja nepreciznosti, linearnosti i netočnosti prema CLSI standardima (engl. *Clinical Laboratory Standards Institute*, CLSI) i smjernicama proizvođača. Pouzdanost mjerenja koncentracije HbA_2 u svrhu određivanja β-talasemije ispitana je na 300 uzoraka bolesnika s prethodno postavljenom dijagnozom β-talasemije.

Rezultati: *Nepreciznost*. Koeficijent varijacije (engl. *coefficient of variation*, CV) unutar serije kod normalne koncentracije HbA_2 (2,2%) iznosio je 1,9%, a kod povišene koncentracije (5,5%) 1,3%. CV iz serije u seriju iznosili su 2,4% (normalna koncentracija) i 1,7% (povišena koncentracija). CV iz dana u dan bili su 3,0% (normalna koncentracija) i 2,7% (povišena koncentracija). Ukupni koeficijenti varijacije bili su 4,4% (normalna koncentracija) i 3,5% (povišena koncentracija). *Linearnost*. $Y = 1{,}089x + 0{,}01$; $R^2 = 0{,}992$ (raspon 2,4–5,0%). Izmjerene su vrijednosti dobro korelirale s očekivanima; prosječno iskorištenje bio je 99%. *Netočnost*. Kod kontrolnog uzorka normalne koncentracije greška je iznosila 1,6% ± 0,26%, a kod kontrolnog uzorka povišene koncentracije 5,2% ± 0,19%. *Referentni raspon* bio je 0,9–3,0% (srednja vrijednost 1,9%, ± 2,5 SD). Svi ispitani nosioci β-talasemije imali su povećanu koncentraciju HbA_2 u krvi [4,7% (3,3–6,9%)].

Zaključak: Analizator Primus Ultra^2 omogućuje brzo i pouzdano razdvajanje različitih frakcija hemoglobina. Mjerenje koncentracije HbA_2 točno je i ponovljivo, što je neophodno zbog male razlike između normalnih i patoloških vrijednosti. Postoji jasna razlika u koncentracijama HbA_2 između zdravih ispitanika i nosioca β-talasemije. Granična se vrijednost za zdravu populaciju može se postaviti na 3,3% HbA_2. Određivanje koncentracije HbA_2 na analizatoru Primus Ultra^2 je pogodna metoda za brzo probiranje unutar populacije na nosioce β-talasemije.

Ključne riječi: HbA_2; probir na β-talasemiju; HPLC

Abstract

Background: Primus Ultra^2 is a HPLC ion exchange analyzer for qualitative and quantitative hemoglobin fraction analysis: in 4 minutes it quantifies HbF, HbA_2, HA_0 and flags abnormal hemoglobins. The aim of the study was to evaluate analytical performance of Primus Ultra^2 analyzer for routine estimation of HbA_2, a critical test to identify β-thalassemia carriers.

Methods: The imimprecision, linearity and inaccuracy studies were performed according to CLSI and manufacturer's guidelines. The reliability of HbA_2 measurements for detection of β-thalassemia was studied by analysis of 300 samples from patients with previous diagnosis of the disease.

Results: *Imprecision*. Within run coefficient of variation (CV) was 1.9% for normal HbA_2 level (2.2%) and 1.3% for elevated level (5.5%). Between run CV was 2.4% for normal level and 1.7% for elevated level. Between-day CV was 3.0% for normal level and 2.7% for elevated level. Total CV was 4.4% for normal level and 3.5% for elevated level. *Linearity*. $Y = 1.089x + 0.01$; $R^2 = 0.992$ (range 2.4–5.0%). The measured values correlated with the expected ones; the mean proportion recovered was 99%. *Inaccuracy*. The mean was 1.6% (SD 0.26%) for normal control and 5.2% (SD 0.19%) for high control. Reference range was 0.9–3.0% (mean 1.9%±2.5 SD). All β-thalassemia carriers had elevated HbA_2 blood concentrations (mean 4.7%, range 3.3–6.9%).

Conclusions: Primus Ultra^2 provides a rapid and reliable separation of different hemoglobins. HbA_2 measurement is accurate and reproducible, which is needed due to the slight difference between normal and pathologic values. There is good differentiation of HbA_2 values between normal subjects and β-thalassemia carriers. The cut off limit can be set at 3.3% HbA_2. This is a suitable method for rapid population screening for β-thalassemia carriers.

Key words: HbA_2 analysis; β-thalassemia screening; HPLC

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Uvod

Otkrivanje i određivanje točne koncentracije frakcija hemoglobina klinički je važno u dva slučaja. Jedan je određivanje točne koncentracije HbA_{1c}, glavnog glikiranog oblika HbA, što je korisno u kliničkoj obradi šećerne bolesti. Metode za određivanje HbA_1c su imunološke ili kromatografske. Drugi je određivanje točne koncentracije HbA_2 i HbF te otkrivanje i određivanje točne koncentracije varijanata hemoglobina, što je glavni postupak u dijagnostici hemoglobinopatija (npr. talasemije ili anemije srpastih stanica).

Beta-talasemija je nasljedni oblik anemije, koja nastaje zbog poremećene sinteze globinskog β-lanca.

Određivanje HbA_2 je najvažniji test za prepoznavanje nositelja β-talasemije, budući da je povećanje ove frakcije hemoglobina najvažnija dijagnostička značajka heterozigotne β-talasemije. HbA_2 je otkriven prije više od 50 godina, te je drugi hemoglobin po redu otkriven u krvi odraslih osoba (1) koji predstavlja oko 3% ukupnog hemoglobina. Kasnija istraživanja pokazala su da je koncentracija HbA_2 povećana kod talasemije (2), te da se HbA_2 sastoji od 2 α- i 2 δ-globinska lanaca, što je navelo Ingrama i Strettona da pretpostave kako se koncentracija HbA_2 u β-talasemiji povećava zbog smanjene sinteze globinskog β-lanca (3). Takvo povećanje nije specifično, budući da su povišene koncentracije nađene i kod drugih stanja (4-6). No, zajedno s eritrocitnim konstantama, HbA_2 ostaje standardni biološki biljeg probira na β-talasemiju u odraslih osoba.

Talasemija je u skupini najčešćih genetskih poremećaja diljem svijeta; 1,7% svjetske populacije su nosioci genetskih promjena povezanih s talasemijom (7). Talasemija je učestala u nekim dijelovima svijeta (mediteranska regija do 8%; zemlje Srednjeg Istoka do 10%; Indija 3–15%; Jugoistočna Azija do 9%), gdje predstavlja veliki javno-zdravstveni problem. No, i u zemljama koje nisu s tog područja, poput zemalja sjeverne Europe i Sjeverne Amerike, bolest se također javlja kao rezultat demografskih promjena uzrokovanih migracijom etničkih manjinskih skupina s visokom učestalošću mutacija povezanih s talasemijom (8). Podaci nedavno provedenog epidemiološkog ispitivanja ukazuju na to da u Europi postoji približno 15.000 osoba s talasemijom *major* koja se javlja kod transfuzije krvi (9-11). Prema tome, određivanje koncentracije HbA_2 igra ključnu ulogu u programima probira na β-talasemiju, budući da povećanje te frakcije hemoglobina omogućuje prepoznavanje heterozigota. Međutim, mogu se pojaviti neke dijagnostičke poteškoće pri identifikaciji atipičnih nosilaca s neznatno povišenim sadržajem HbA_2. Pojava tih graničnih slučajeva nije rijetka zbog velike heterogenosti genotipova β-talasemije ili moguće istodobne prisutnosti drugih stečenih ili genetskih stanja (npr. manjak željeza, δ-talasemija) koja mogu prikriti dijagnozu heterozigotne β-talasemije (12-14). Stoga je, zbog teškog razlikovanja iz-

Introduction

Detection and quantification of hemoglobin (Hb) fractions is clinically relevant in two situations. First, quantification of HbA_{1c}, the major glycated form of HbA, is useful in the clinical management of diabetes. Methods for HbA_{1c} analysis include immunoassay or chromatography. Second, the quantification of HbA_2 and HbF, and the detection and quantification of Hb variants is an essential tool in the diagnosis of hemoglobinopathies (e.g., thalassemia or sickle cell syndromes).

β-thalassemia is an inherited form of anemia resulting from defective synthesis of β-globin chain. HbA_2 is a critical test to identify β-thalassemia carriers because the increase in this Hb fraction is the most important diagnostic characteristic of heterozygous β-thalassemia.

HbA_2 was identified more than 50 years ago as the second Hb in the blood of healthy adults (1), representing about 3% of total Hb. Later studies demonstrating HbA_2 to be increased in thalassemia trait (2) and to comprise 2 α and 2 δ globin chains, led Ingram and Stretton to postulate that, because β globin synthesis was decreased, an increase of HbA_2 was likely in β-thalassemia (3). Such an increase is not specific because elevated concentrations have also been reported in other conditions (4-6). In conjunction with erythrocyte indices, however, HbA_2 remains a standard biomarker for β-thalassemia screening in adults.

Thalassemia syndromes are among the most common genetic disorders worldwide, with 1.7% of the world's population carrying thalassemic genes (7). Thalassemia is prevalent in some parts of the world (Mediterranean region, up to 8%; Middle East countries, up to 10%; India, 3%–15%; and Southeast Asia, up to 9%) where it poses a major public health problem. However, non-endemic countries such as northern Europe and North America are also involved in thalassemia related problems as the result of demographic changes caused by migration of ethnic minority groups with a high frequency of thalassemic mutations (8). Data from recent epidemiological surveys indicate that in Europe there are approximately 15,000 subjects with transfusion dependent thalassemia major (9-11). In this regard, HbA_2 determination plays a key role in screening programs for β-thalassemia, since an increase in this Hb fraction allows for heterozygotes to be recognized. However, some diagnostic difficulties may arise on identification of atypical carriers having slightly increased HbA_2 values. The finding of such borderline subjects is not rare because of the great heterogeneity of β-thalassemia genotypes or the possible coexistence of other acquired or genetic conditions (i.e. iron deficiency, δ-thalassemia) that may mask the diagnosis of heterozygous β-thalassemia (12-14). Therefore, owing to the narrow separation between normal and pathological HbA_2 values, strict analytical quality of HbA_2 measureme-

među normalnih i patoloških koncentracija HbA_2, za točnu dijagnozu ključna visoka analitička kvaliteta mjerenja koncentracije HbA_2, pogotovo pri genetskom savjetovanju kod kojeg se moraju identificirati parovi s povećanim rizikom od nastanka bolesti kod potomstva.

Metode za ispitivanje hemoglobinopatija tradicionalno uključuju alkalnu i kiselu elektroforezu hemoglobina u kombinaciji s određivanjem točne koncentracije HbA_2 (elektroforeza ili kromatografija) i HbF (alkalna denaturacija, elektroforeza ili kromatografija). Sve se nabrojene metode izvode ručno i dugotrajne su. Posljednjih desetljeća razvijaju se automatizirane tehnike za određivanje frakcija hemoglobina, koje kliničarima daju zadovoljavajuće rezultate, te poboljšavaju djelotvornost laboratorija i kvalitetu rezultata.

Tekućinska kromatografija visoke djelotvornosti (HPLC) kationske izmjene potpuno je automatiziran sustav kvalitativne i kvantitativne analize frakcija hemoglobina, pogodan za laboratorije s velikim obujmom posla (16,17). Uzorci se ubrizgavaju u kromatograf opremljen kolonom za izmjenu iona, koja je uravnotežena prema pH i ionskoj snazi. Odvajanje vrsta hemoglobina postiže se upotrebom gradijenta između dviju mobilnih faza koje se razlikuju po koncentraciji soli i pH. Fizičke značajke kao što su promjene na površini te prisutnost hidrofilnih i hidrofobnih skupina određuju stopu migracije svake vrste hemoglobina. U usporedbi sa zahtjevnim konvencionalnim tehnikama, HPLC metode imaju znatne prednosti, kao što su precizno određivanje koncentracije, ušteda vremena i potpuna automatizacija. Danas HPLC tehnike predstavljaju najpouzdanije metode za razdvajanje, identifikaciju i određivanje koncentracije frakcija hemoglobina, ali imaju i potencijalna ograničenja poput lažne interpretacije u prisutnosti patoloških hemoglobina.

Materijali i metode

HPLC sustav Primus Ultra^2

Analizator Primus Ultra^2 (Primus Corporation, Kansas City, Kansas, SAD) rabi načela HPLC ionske izmjene i spektrofotometrijskog otkrivanja. Sustav je načinjen tako da pruži najveću moguću količinu podataka u najkraćem vremenu. Ova metoda kombinira osjetljivost i specifičnost HPLC s automatskim programom za obradu i pruža pomoć u procjeni podataka.

Uzorci se obrađuju u serijama i svaka serija započinje FASC kontrolom kao biljegom retencijskih vremena za poznati hemoglobin koji sadrži: HbF, HbA_0, HbC i HbS, kako mu ime FASC govori (slike 1. i 2.). FASC kontrola osigurava optimalan rad sustava. Kao uzorci su prikladne i puna i hemolizirana krv.

Pet mikrolitara pune krvi automatski se sakuplja, hemolizira i ubrizgava u kolonu kojom protječe mješavina pufera

nt is an essential requirement for accurate diagnosis, particularly for genetic counseling when couples at risk have to be identified.

The methods for hemoglobinopathy investigation traditionally include alkaline and acid Hb electrophoresis in combination with HbA_2 quantification by electrophoresis or chromatography, and HbF quantification with these methods or alkali denaturation. Above mentioned methods are manual and time consuming (15). In the last decades, automated techniques have been developed for Hb fractionation, producing results required by clinicians, while improving laboratory efficiency and quality of results.

Cation exchange high performance liquid chromatography (HPLC) is a fully automated system for qualitative and quantitative Hb fraction analysis, suitable for laboratories with a high workload (16,17).

Samples are injected into a chromatograph equipped with an ion-exchange column that has been equilibrated with respect to pH and ionic strength. Separation of Hb species is accomplished through the use of a gradient between two mobile phases with differences in salt concentration and pH. Physical characteristics such as surface charge and the presence of hydrophilic and hydrophobic groups determine the rate of migration of each species. HPLC methods have considerable advantages over labor-intensive conventional techniques in terms of precise quantification, time savings and full automation. Currently, HPLC techniques are the most reliable methods for separation and quantification of Hb fractions, and are useful tools in presumptive identification of Hb variants but having potential limits of false interpretation in the presence of an abnormal Hb.

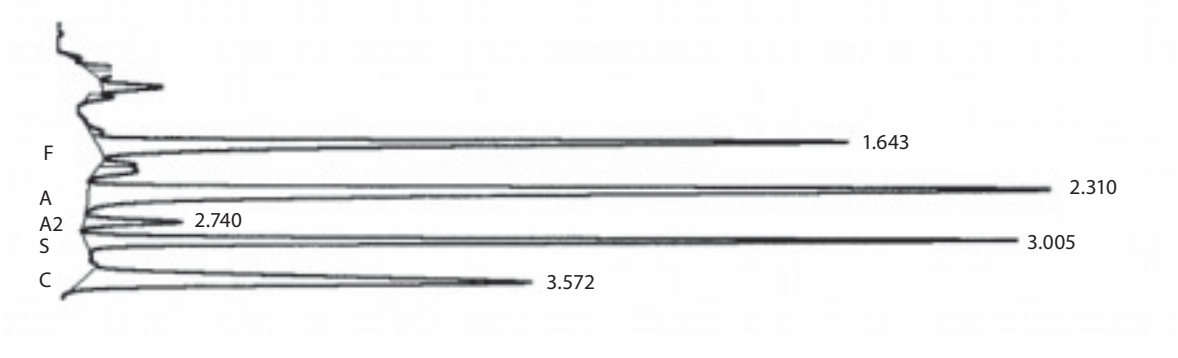
Materials and Methods

Primus Ultra^2 HPLC system

Primus Ultra^2 analyzer (Primus Corporation, Kansas City, Kansas, USA) employs principles of ion exchange chromatography HPLC and spectrophotometric detection. The system has been designed to provide the maximum amount of information in the shortest time. The method combines the sensitivity and specificity of HPLC with automation software and provides assistance in data evaluation.

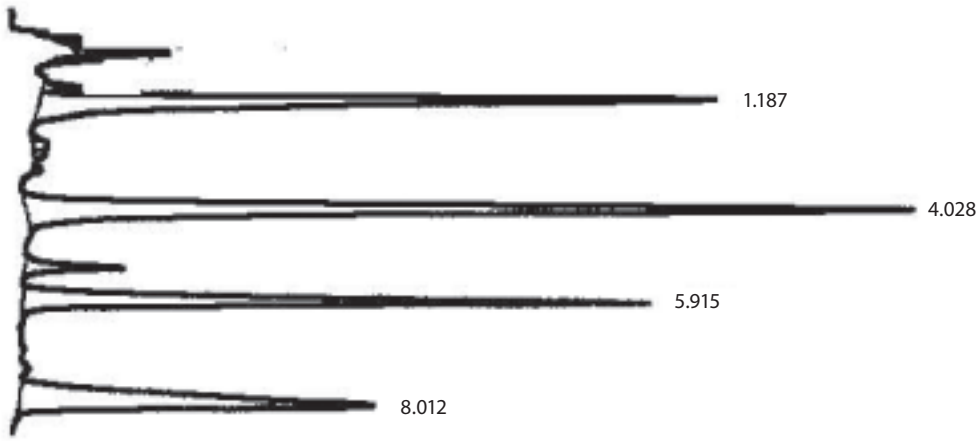
Samples are run in batches and every run starts with a FASC control as a retention time marker for the known hemoglobins that it contains: HbF, HbA_0, HbS and HbC, as stated by the name FASC (Figures 1 and 2). FASC control ensures optimal system performance. Both whole blood and hemolysates are suitable samples.

Five µL of whole blood are automatically sampled, hemolyzed and injected onto the column during the flow



Slika 1. Kromatogram PRIMUS Ultra², FASC kontrola, metoda brzog probira (Quick Scan assay). Metoda brzog probira: za probiranje uzoraka bolesnika. Vrijeme analize iznosi 4 minute, sustav određuje koncentraciju HbF, HbA_2, HbA_0 i označuje vrške drugih vrsta hemoglobina; FASC kontrola je biljeg retencijskih vremena za poznate hemoglobine: HbF, HbA_0, HbC i HbS.

Figure 1. Primus Ultra² chromatogram, FASC control Quick Scan assay. Quick Scan assay: for screening patient samples. Analysis time is 4 minutes; the system quantifies HbF, HbA_2, HbA_0 and flags abnormal peaks. FASC control is a retention time marker for the known hemoglobins it contains: HbF, HbA0, HbC and HbS.



Slika 2. Kromatogram PRIMUS Ultra², FASC kontrola, metoda visoke rezolucije (High Resolution assay). Metoda visoke rezolucije: u 10,5 minuta rastavlja druge vrste hemoglobina kao pomoć pri njihovoj identifikaciji.

Figure 2. Primus Ultra² chromatogram, FASC control high resolution assay. High resolution assay: in 10.5 minutes it further resolves abnormal hemoglobins to aid in their identification.

(mobilne faze 1 i 2). Nakon ispiranja s kolone sastavnice uzorka prolaze kroz spektrofotometrijski detektor gdje se na valnoj duljini od 413 ± 2 nm otkrivaju pojedine frakcije. Postoje dvije metode analize: metoda brzog probira (engl. *Quick Scan Method*) – za probir uzoraka bolesnika. Vrijeme analize je 4 min, sustav određuje koncentraciju HbF, HbA_2, HbA_0 i označuje vrijednosti izvan granica normale. Metoda visoke rezolucije (engl. *High Resolution Method*): u 10,5 minuta rastavlja druge vrste hemoglobina kao pomoć pri identifikaciji. Ovom metodom može se prepoznati i identificirati i do 100 varijanata hemoglobina. Svaka se metoda može rabiti za određivanje točne koncentracije HbA_2 i HbF ukoliko se u seriji odrede i kontrolni uzorci.

of appropriately blended buffers (mobile phases 1 and 2). Upon elution from the column, sample components pass through the spectrophotometric detector, where detection occurs at a wavelength of 413 ± 2 nm.
Two analysis methods are available, i.e. Quick Scan assay for screening patient samples. Analysis time is 4 minutes; the system quantifies HbF, HbA_2, HbA_0 and flags abnormal peaks; and High Resolution assay: in 10.5 minutes it further resolves abnormal hemoglobins to aid in their identification. Up to 100 Hb variants can be recognized and identified with this high resolution working method. Either program may be used to quantify HbA_2 and HbF if controls have been run with the batch.

Istodobno dok se uzorak analizira, na monitoru se prikazuje kromatogram u stvarnom vremenu. Računalo tiska izvještaj s informacijama o uzorku, datumom i vremenom, nakon čega slijedi kromatogram s retencijskim vremenima označenima kod svakog vrška. Na kraju se tiska objedinjen izvještaj za svaki vršak s apsolutnim i relativnim retencijskim vremenom, postotkom površine i komentarima za svaki vršak u kromatogramu.
Cilj ovoga istraživanja bila je ocjena analitičkog rada i kvalitete rezultata dobivenih sustavom Primus Ultra² za rutinsko određivanje HbA_2 i probir na β-talasemiju.

Bolesnici

U istraživanje je bilo uključeno 150 uzoraka krvi naizgled zdravih ispitanika, dobivenih iz Zavoda za transfuziju krvi, kako bi se odredio referentni raspon, prema standardima CLSI. Kompletna krvna slika i parametri metabolizma željeza bili su unutar referentnog raspona. Pouzdanost mjerenja koncentracije HbA_2 sustavom Primus Ultra² za otkrivanje β-talasemije određena je analizom 300 uzastopnih uzoraka odraslih bolesnika s prethodno dijagnosticiranom β-talasemijom (53% muškaraca, 47% žena; raspon dobi 18–95 godina, srednja dob 49 godina). U tablici 1. prikazane su vrijednosti biokemijskih parametara za zdrave ispitanike i ispitanike s talasemijom. Na slici 3. prikazan je kromatogram nosioca β-talasemije.
Probir na β-talasemiju obavljen je rutinski u našem laboratoriju mjerenjem eritrocitnih konstanti i koncentracije HbA_2. Molekularna karakterizacija mutacija obavljena je tehnikom PCR-ASO (engl. *polymerase chain reaction – allele-specific oligonucleotide hybridization*, PCR-ASO).
Slijedeći naš protokol, dijagnosticirali smo skupinu nosioca β-talasemije: eritrocitne konstante određene su analizatorom LH750 Beckman-Coulter (Beckman Coulter, Inc., Miami, FL, SAD).

As the sample is analyzed, a chromatogram is displayed on the monitor in real time. The computer produces printed reports with sample identification information, date and time, followed by a chromatogram with retention times indicated at the apex of each peak. A peak summary report is printed after the chromatogram with retention time, relative retention time, % area and comments for each peak in the chromatogram.
The aim of the study was to evaluate analytical performance and quality of results obtained with the Primus Ultra² system for routine estimation of HbA_2 and screening for β-thalassemia.

Patients

One hundred and fifty samples from apparently healthy subjects obtained from the Blood Transfusion Department were used to establish the reference range, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Blood cell counts and biochemical iron test results were within the reference ranges.
The reliability of HbA_2 measurement by Primus Ultra² for detection of β-thalassemia was assessed by analyzing 300 samples from consecutive adult patients with a previous diagnosis of the disease (53% male and 47% female, age range 18-95, mean age 49 years). Table 1 shows analytical data of study patients and Figure 3 a β-thalassemia carrier chromatogram.
Screening for β-thalassemia is routinely performed in our laboratory by measuring red blood cell indices and level of HbA_2. Molecular characterization of mutations is performed with the PCR-ASO (polymerase chain reaction-allele-specific oligonucleotide hybridization) technique.
The group of β-thalassemia carriers was diagnosed following our protocol: red blood cell indices were determined on an LH750 Beckman-Coulter (Beckman Coulter

Slika 3. Kromatogram PRIMUS Ultra², nosilac β-talasemije. Kromatogram s istaknutim retencijskom vremenima kod svakog vrška. Nakon kromatograma ispisuje se objedinjen izvještaj za svaki vršak s apsolutnim i relativnim retencijskim vremenom, postotkom površine i komentarima za svaki vršak u kromatogramu.

Figure 3. Primus Ultra² chromatogram, β-thalassemia carrier. Chromatogram with retention times indicated at the apex of each peak. A peak summary report is printed after the chromatogram with the retention time, relative retention time, % area and comments for each peak in the chromatogram.

Tablica 1. Hematološki i biokemijski parametri referentne skupine i kod 300 ispitanika s β-talasemijom.

Table 1. Hematological and biochemical parameters in the reference group and in a pool of 300 β-thalassemia patients.

	RBC 10^{12}/L	Hb g/dL	MCV fL	MCH pg	MCHC g/dL	HbA_2 %	HbF %	Iron µmol/L	Transferrin g/L	Ferritin µg/L	Sat %
Referent group	4.9 ± 0.27	15.4 ± 0.64	91.1 ± 2.55	31.3 ± 1.53	34.3 ± 0.52	1.9 ± 0.71	0.3 ± 0.3	16.5 ± 0.62	2.53 ± 0.2	75 ± 2.8	31 ± 1.9
β-thalassemia	5.8 ± 0.43	12.6 ± 1.21	63.8 ± 3.74	21.5 ± 1.3	33.7 ± 0.92	4.7 ± 0.7	1.4 ± 0.4	18.1 ± 3.95	2.48 ± 0.28	137 ± 125	30 ± 5.6

RBC – red blood cell count; Hb – hemoglobin; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; % sat – % transferrin saturation.

Uzorci s eritrocitozom (RBC > 5,8 x 10^{12}/L) i mikrocitozom (MCV < 70 fL) odabrani su za određivanje koncentracije HbA_2 na HPLC analizatoru HA 8160 Menarini (Menarini Diagnostics, Firenca, Italija).
Kod bolesnika s koncentracijom HbA_2 višom od referentne vrijednosti (3,5%) postoji klinička sumnja na β-talasemiju te su njihovi uzorci poslani na molekularnu analizu.
DNA je dobivena pročišćavanjem na kolonama pomoću Generation^R Capture Column kita (Gentra Systems, Minneapolis, MN, SAD).
Molekularna analiza otkrivanja prisutnosti najčešće mediteranske točkaste mutacije obavljena je setom testova mDxR BeTha Gene 1 (Bio Rad Laboratories, Hercules, CA, SAD), prema načelu ASO hibridizacije.
U tablici 2. prikazane su ispitane mutacije te rezultate dobiveni za 300 nosioca β-talasemije. Svi su bili heterozigoti.

Analitička procjena

Netočnost

Netočnost se određivala analizom dvaju komercijalnih kontrolnih uzoraka po seriji u više od 20 dana. Izračunate su srednja vrijednost, standardna devijacija te koeficijent varijacije.

Inc. Miami, FL, USA) analyzer. Samples with erythrocytosis (red blood cell (RBC) > 5.8 x 10^{12}/L) and microcytosis (MCV < 70 fL) were selected for HbA_2 quantification, performed by HPLC HA 8160 Menarini (Menarini Diagnostics, Florence, Italy). HbA_2 over the reference value of 3.5% strongly suggests β-thalassemia trait. These samples underwent molecular analysis. DNAs were purified with Generation^R capture column kit (Gentra Systems, Minneapolis, MN, USA).
Molecular analysis for the presence of the most common Mediterranean point mutations were performed with mDx^R BeTha Gene 1 (Bio Rad Laboratories, Hercules, CA, USA), based on the principle of ASO hybridization. Table 2 resumes the mutations studied, the mutant class and the results obtained for the series of 300 β-thalassemia carriers. All of them were found to be heterozygous.

Analytical evaluation

Inaccuracy

Inaccuracy was assessed by analyzing two commercially available control samples *per* run over 20 days. The mean, standard deviation (SD) and coefficient of variation (CV) were calculated.

Tablica 2. Distribucija mutacija β-talasemije izraženih kao postotak u ukupnom broju ispitanika (300 nosioca β-talasemije)

Table 2. Distribution of β-thalassemia mutations expressed as percentage of the total number studied (300 β-thalassemia carriers)

Mutations	Mutant Class	Frecuency %
CD39 (C-T)	Non functional mRNA	42.9
IVS1: 110 (G-A)	RNA processing mutant	30.6
IVS1: 6 (T-C)	RNA processing mutant	12
IVS1:1 (G-A)	RNA processing mutant	12
IVS2: 745 (C-G)	RNA processing mutant	1.3
IVS2: 1 (G-A)	RNA processing mutant	0
-87 (C-G)	Transcriptional mutant	0
CD6 – A	Non functional mRNA	1.3

Nepreciznost u seriji

Dva su uzorka (jedan zdravog ispitanika, drugi nosioca β-talasemije) analizirana 10 puta unutar iste serije te su izračunate srednje vrijednosti, SD i CV.

Nepreciznost iz dana u dan

Dva su uzorka testirana 3 puta 7 dana za redom, te su izračunate srednja vrijednost, SD i CV dnevnih srednjih vrijednosti za tih 7 dana.

Ispitivanje iskorištenja

Ispitivanje iskorištenja miješanjem uzoraka načinjeno je kako bi se ocijenila sposobnost otkrivanja povećane koncentracije HbA_2 analizatora Primus Ultra^2. Normalna krv (2,4% HbA_2) i uzorak krvi nosioca β-talasemije (5,0% HbA_2) pomiješani su u omjerima 9:1, 8:2…1:9 te je analizirano jedanaest uzoraka. Za usporedbu očekivanih i izmjerenih vrijednosti napravljen je linearni regresijski test (statistički program Method Validator 1.15, P. Marquis, Metz, Francuska).

Rezultati

Referentni raspon

Srednja vrijednost koncentracije HbA_2 u krvi bila je 1,9%, a referentni interval 0,9–3,0% (srednja vrijednost ± 2,5 SD). Svi ispitani nosioci β-talasemije imali su povišenu koncentraciju HbA_2 u krvi, uz raspon vrijednosti od 3,3% do 6,9% i srednju vrijednost 4,7%.

Netočnost

Netočnost je određena analizom dvaju komercijalnih kontrolnih uzoraka po seriji u vremenu od preko 20 dana. Izračunate su srednja vrijednost i SD dobivenih rezultata, koje su iznosile 1,6% ± 0,26% za kontrolni uzorak normalne koncentracije i 5,2% ± 0,19% za kontrolni uzorak povišene koncentracije.

Nepreciznost

Nepreciznost je ispitana obradom uzoraka s normalnim i visokim koncentracijama HbA_2. Preciznost unutar serije: dva uzorka (jedan zdravog ispitanika, drugi nosioca β-talasemije) analizirani su 10 puta unutar iste serije te su se izračunate srednje vrijednosti, SD i CV. Preciznost iz dana u dan: dva uzorka (jedan zdravog ispitanika, drugi nosioca β-talasemije) testirani su više od 7 dana za redom, 3 puta svakog dana. Uzorci su pohranjeni na 4 °C. Za svaki je dan izračunata srednja vrijednost i CV dnevne srednje vrijednosti. CV unutar serije bio je 1,9% kod normalne koncentracije HbA_2 (2,2%) i 3,3% kod povišene koncentracije HbA_2 (5,5%). CV iz serije u seriju bio je nešto viši i iznosio je 2,4% (normalna koncentracija) i 1,7% (povišena koncentracija). CV iz dana u dan bio je 3,0% (normalna koncentracija) i 2,7% (povišena koncentracija).

Intra-assay imprecision

Two samples (one from a healthy adult and another from a β-thalassemia carrier) were analyzed 10 times within the same run. The mean, SD and CV were calculated.

Inter-assay imprecision

Two samples were assayed 3 times each day, over seven days. Samples were stored at 4 °C every day. The mean, SD and CV of daily mean values were calculated for 7 days.

Recovery study

Recovery study by sample mixing was performed to evaluate the response of Primus Ultra^2 to increasing concentrations of the analyte, i.e. HbA_2.
A normal blood (2.4% HbA_2) and β-thalassemia carrier sample (5.0% HbA_2) were mixed at the ratios of 9:1, 8:2… 1:9 and eleven samples were analyzed.
To make comparison between the theoretical expected values and measured ones, a linear regression test was performed (Method Validator 1.15, P. Marquis statistical software, Metz, France).

Results

Reference range

The mean HbA_2 blood concentration was 1.9% and reference range 0.9–3.0% (mean ± 2.5 SD). All β-thalassemia carriers studied had increased HbA_2 blood concentration, the values ranging from 3.3% to 6.9%, mean 4.7%.

Inaccuracy

Inaccuracy was assessed by analyzing two commercially available control samples *per* run over 20 days. The mean and standard deviation (SD) were calculated. The following results were obtained: 1.6% (SD 0.26) for normal control and 5.2% (SD 0.19) for high control.

Imprecision

Imprecision was tested by running samples with normal and high HbA_2 concentrations. Intra-assay imprecision: two samples (one from a healthy adult and another from a β-thalassemia carrier) were analyzed 10 times within the same run. The mean and CV were calculated. Inter-assay imprecision: two samples (one from a healthy adult and another from a β-thalassemia carrier) were assayed 3 times each day, for seven days. Samples were stored at 4 °C every day. The mean and CV of daily mean values were calculated for 7 days. Within-run CV was 1.9% for normal HbA_2 level (2.2%) and 1.3% for elevated HbA_2level (5.5%). Between-run CVs were slightly higher, i.e. 2.4% (normal level) and 1.7% (elevated level). Between-day CVs were 3.0% and 2.7% for normal and high HbA_2 concentrations,

Ukupni koeficijent korelacije bio je 4,4% (normalna koncentracija) i 3,5% (povišena koncentracija).

Ispitivanje iskorištenja

Ispitivanje iskorištenja miješanjem uzoraka napravljeno je kako bi se ocijenila sposobnost otkrivanja povećanih koncentracija analita HbA_2 za analizator Primus Ultra^2. Normalna krv (2,4% HbA_2) i uzorak nosioca β-talasemije (5,0% HbA_2) pomiješani su u omjerima 9:1, 8:2…1:9 i jedanaest je uzoraka analizirano.
Za usporedbu teoretski očekivanih i izmjerenih vrijednosti napravljen je linearni regresijski test (statistički program Method Validator 1.15, P. Marquis, Metz, Francuska). Najbolji pravac i pravac linearne regresije dobiven za iskorištenje bio je: $y = 1,089x + 0,01$, $R^2 = 0,992$.
Izmjerene vrijednosti bile su u dobroj korelaciji s očekivanima i prosječan omjer iskorištenja bio je 99% (tablica 3.).

respectively. Total CVs were 4.4% (normal level) and 3.5% (elevated level).

Recovery study

Recovery study by sample mixing was performed to evaluate the response of Primus Ultra^2 to increasing concentrations of the analyte, i.e. HbA_2.
A normal blood (2.4% HbA_2) and β-thalassemia carrier sample (5.0% HbA_2) were mixed at the ratios of 9:1, 8:2… 1:9 and eleven samples were analyzed.
To make comparison between the theoretical expected values and measured ones, a linear regression test was performed (Method Validator 1.15, P. Marquis statistical software, Metz, France). The line of best fit and linear regression obtained for recovery was $Y = 1.089x + 0.01$; $R^2 = 0.992$. The measured values correlated well with the expected ones, and the average proportion recovered was 99% (Table 3).

Tablica 3. Rezultati istraživanja iskorištenja

Table 3. Recovery study results

Theoretical value %	Observed value %	Recovery %
2.66	2.7	101
2.92	2.8	96
3.18	3.1	97
3.44	3.3	96
3.7	3.8	102
3.96	3.9	98
4.22	4.4	103
4.48	4.6	102
4.74	4.8	101

Rasprava

Iako je talasemija globalno najzastupljenija na Mediteranu i zemljama Dalekog Istoka, zbog populacijskih migracija doslovce ne postoji zemlja na svijetu u kojoj barem mali postotak stanovnika ne boluje od talasemije. Kao i za sve kronične bolesti tako je i u slučaju talasemije važna cjelovita obrada bolesti: odgovarajući probir, otkrivanje bolesnika i savjetovanje parova s povećanim rizikom bitni su postupci za smanjenje pobola i smrtnosti bolesnika (18).
β-talasemija se sa sigurnošću može dijagnosticirati u slučaju povišene koncentracije HbA_2, broja eritrocita većeg od 5×10^{12}/L, mikrocitoze i normalne koncentracije feritina u serumu. Iako se dobivene vrijednosti koncentracije

Discussion

Although thalassemia is most common in the Mediterranean basin and Far East countries, due to migration of populations there is virtually no country in the world now in which thalassemia does not affect some percentage of the population. Like other chronic diseases, prevention is important in the overall management of the disease, whereas appropriate screening, detection of patients and counseling of couples at risk are the most important procedures to reduce the morbidity and mortality rates of the disease (18).
β-thalassemia can be diagnosed with confidence when elevated HbA_2, RBC count over 5×10^{12}/L, microcytosis and normal serum ferritin are present. Although HbA_2

HbA_2 trebaju interpretirati zajedno s ostalim parametrima, npr. koncentracijom željeza, eritrocitnim konstantama ili obiteljskom anamnezom, zbog kliničke važnosti određivanja HbA_2 u probiru na β-talasemiju i ozbiljnih posljedica uslijed netočne dijagnoze, dobiveni rezultati moraju biti visoke kvalitete. Imajući to na umu treba napomenuti kako još ne postoje konačno definirani analitički ciljevi mjerenja koncentracije HbA_2. Ipak, dobro je da se rabe sastavnice biološke varijacije kako bi se utvrdile specifikacije analitičke kvalitete za preciznost, sustavnu i ukupnu pogrešku kliničko-laboratorijskih postupaka. Intra- i inter-individualne sastavnice varijacije izražene kao koeficijenti varijacije (CVw, intra-individualne i CVg, inter-individualne) rabe se za izračunavanje ukupne pogreške (engl. *total error*, TE) prema formuli:

$$TE = 1{,}65 \times CVa + \frac{\sqrt{CV^2w + CV^2g}}{4}$$

gdje je cilj preciznosti postavljen na osnovi intra-individualne varijabilnosti CVa < 0,5 CVw (19). Pretpostavljajući da intra-individualna varijabilnost koncentracije HbA_2 može biti slična koncentraciji HbA_0 (CVI između 2,8% i 3,4%) (20), analitički cilj za preciznost može se utvrditi između 1,4% i 1,7%. Zbog te inter-individualne varijabilnosti znanstvena literatura još ne nudi opširnije podatke. Gruba procjena može se ekstrapolirati iz referentnog raspona gdje se izračuna prosječna koncentracija HbA_2 u zdravih odraslih osoba i time se dobiva ukupna dozvoljena pogreška od 7,8% (21).

Važno je naglasiti da referentni raspon dobiven u ovoj ocjeni (srednja vrijednost 1,9%, raspon ± 2,5 SD 0,9–3,0%), slično rasponu što ga navodi proizvođač (srednja vrijednost 1,9%, raspon ± 2,5 SD 0,7–3,1%), ima niže vrijednosti nego što se mogu dobiti na drugim HPLC sustavima (srednja vrijednost 2,5%, raspon ± 2,5 SD 2,5–3,5%) (22). Ove bi se vrijednosti mogle objasniti niskom procjenom sustava integriranja površine vršaka. Ukupna dozvoljena pogreška ekstrapolirana iz referentnog raspona 0,9–3,0%, CVg 15,2%, iznosi 6,5%. Ukupni koeficijenti varijacije dobiveni ispitivanjem preciznosti niži su od ove granice. U pogledu ponovljivosti testa HbA_2, analizator Tosoh G7 je postigao dobru preciznost unutar serije, malo više od željenog cilja testa i nešto višu preciznost između serija.

Sustav Primus Ultra^2 pruža brzo i pouzdano razdvajanje i određivanje relativnog postotka različitih tipova hemoglobina, osobito HbA_2. Dobiveni su rezultati točni i ponovljivi. Omogućeno je dobro razlikovanje zdravih osoba i nosioca β-talasemije. Granična se vrijednost može postaviti na 3,3%, s time da se svi bolesnici s vrijednostima višim od 3,3% identificiraju kao nosioci β-talasemije.

Lakoća rada i potreba ograničenog tehničkog znanja čine ovaj analizator pogodnim za laboratorije s velikim obujmom posla, poboljšavajući njihovu djelotvornost i sma-

concentrations obtained should be interpreted together with other parameters such as iron status, erythrocyte indices or family studies, due to the clinical value of HbA_2 assay in the screening for β-thalassemia and the serious consequences of an incorrect diagnosis, the results obtained must be of high quality. With this in mind, it is important to note that no analytical goals for HbA_2 measurements have been definitely defined. It is, however, well recognized that the components of biological variation have been used to derive analytical quality specifications for the imprecision, bias and total error of clinical laboratory procedures. The within- and between-subject components of variation, expressed in terms of coefficients of variation (CVw and CVg, respectively) are used to calculate the total error (TE) according to the formula:

$$TE = 1.65 \times CVa + \frac{\sqrt{CV^2w + CV^2g}}{4}$$

where the goal for imprecision is set on the basis of intra-individual variability CVa < 0.5 CVw (19). Assuming that the intra-individual variation of HbA_2 could be similar to that of HbA_0 (CV_I between 2.8% and 3.4%) (20), the analytical goal for imprecision can be defined between 1.4% and 1.7%. With regard to inter-individual variability, no data are yet available in the literature. A raw estimate could be extrapolated from the reference range in which an average HbA_2 can be calculated in normal adults, thus producing a total allowable error of 7.8% (21).

It is important to state that the reference range established in this evaluation (mean 1.9%, range ±2.5 SD 0.9–3.0%), similar to the range provided by the manufacturer (mean 1.9%, range ± 2.5 SD 0.7–3.1%) has lower limits than the range obtained on other HPLC systems (mean 2.5%, range ± 2.5 SD 2.5–3.5%) (22). These values could be explained by the underestimation of the system integrating peak area.

Total allowable error extrapolated from for the reference range 0.9–3.0%, CV_g 15.2%, is 6.5%. Total CVs obtained in the imprecision study are lower than this limit.

With respect to HbA_2 reproducibility, the Tosoh G7 analyzer was found to yield good within-run imprecision, just a little above the desirable goal for the assay, and a slightly higher between-run imprecision.

The Primus Ultra^2 system provides a rapid and reliable separation and determination of the relative percentage of different hemoglobin types, particularly HbA_2. The results obtained are accurate and reproducible. There is good differentiation of HbA_2 values between normal patients and β-thalassemia carriers. The cut off limit can be set at 3.3%, with all patients with values higher than 3.3% being identified as β-thalassemia carriers.

The ease of the operation and the limited technical work make this analyzer suitable for laboratories with a high workload, improving its efficiency and allowing the cost

njujući troškove probira na talasemiju i hemoglobinopatije. Naše istraživanje je pokazalo kako je ova metoda primjerena za brz probir populacije na nositelje β-talasemije i pouzdana za određivanje točne koncentracije HbA_2 u krvi. Stoga se smatra prikladnom za rutinsku kliničko-laboratorijsku upotrebu, no, kao što je to slučaj sa svim laboratorijskim testovima prvoga stupnja za karakterizaciju varijanata hemoglobina, ovaj HPLC sustav ima potencijalno ograničenje lažne interpretacije vršaka u prisutnosti patoloških hemoglobina. U tim se slučajevima preporučaju laboratorijski testovi drugoga stupnja, npr. molekularna analiza ili obiteljska ispitivanja za konačnu identifikaciju varijante hemoglobina.

of thalassemia and hemoglobinopathy screening to be reduced.

Our study showed the method to be appropriate for rapid population screening for β-thalassemia carriers, reliable for quantitative determination of HbA_2 in blood and suitable for routine clinical laboratory use; however, as in any of the first-level laboratory tests for the characterization of Hb variants, this HPLC system also has potential limits of false interpretation in the presence of an abnormal Hb peak. In these cases, it is recommended that second-level laboratory tests such as molecular analysis and family studies be undertaken in order to achieve definitive identification of the Hb variant.

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