lated concentrations [mean (SD) differences, 0.6 (4.5%) 0.7 (3.9%), 0.2 (13%), and 0.8 (9.6%) for renal, heart, lung cystic fibrosis, and lung non-cystic fibrosis transplant patients, respectively] and good precision (root mean squared error, 1.1–5.7%).

The calculated RCE values at each studied sampling time for each profile for the five different populations are shown in Fig. 1. For each transplant patient, whatever the type of graft, the RCE value was within ±20% in a sampling-time interval from 110 to 130 min. A sampling-time error of ±15 min produced a RCE >20% (up to 30%) in a few heart and lung transplant patients. As can be inferred from Fig. 1, in many patients t_{max} was later than c_{2}, which could be designated as “delayed absorption”. The present data set included patients with a t_{max} up to 5 h. In renal transplant patients, overestimation of c_{2} was observed for t<120 min and underestimation for t>120 min. In lung and heart transplant patients, over- as well as underestimation of c_{2} could be observed regardless of the time error, depending on the patient.

On the basis of data obtained in renal, heart, and lung transplant patients and using a validated pharmacokinetic method, the present study shows that when the sampling-time error around 2 h post dose increases, the relative concentration error and its interindividual variability also increase significantly. Although the impact of sampling-time error differed with the type of graft, an acceptable (+20%) estimation of the true c_{2} value was obtained within a time-error range of ±10 min for all 124 profiles. Interestingly, for renal transplant patients, the mean RCE was always <10% within this time range.

The c_{2} target values defined for CsA TDM in renal and de novo liver transplant patients have been proposed with a range of ±20% (e.g., 1.7 mg/L ±20% for the 0–1 month post-transplantation period in renal transplantation) [4]. We compared this range with the interlaboratory CV values of the International Cyclosporin Proficiency Testing Scheme, taken as estimates of the analytical error [15]. For concentrations <500 μg/L (n = 12; results for the year 2002), the mean CVs were 10% for the Emit and 6.0% for the FPIA. The interlaboratory CV obtained with a whole blood sample to which 2000 μg/L cyclosporin had been added was 7.9% for the Emit (n = 38) and 7.2% for the FPIA (n = 30).

In summary, numerous studies have promoted a Neoral monitoring strategy using CsA blood concentrations measured 2 h after drug administration, called c_{2}, to improve the clinical benefits for transplant patients. Guidelines for c_{2} interpretations propose target values with a range of ±20%. The present study shows that the accuracy of c_{2} monitoring is highly dependent on the correct sampling time and that a substantial difference (up to 30%) from the 2-h values (which are themselves subject to analytical inaccuracy and imprecision) can occur with a sampling-time error of 15 min. Consequently, such time errors could lead to inappropriate dose adjustment and to inadequate immunosuppression or increased risk of adverse effects. Timing errors of ±10 min seem to be the acceptable limit for use of c_{2} and subsequent dose adjustment of CsA.

References


Denaturing HPLC Procedure for Factor IX Gene Scanning, Giuseppe Castaldo,1,2 Paolo Nardello,1 Fabiana Bellitti,1 Angiola Rocino,1 Antonio Coppola,4 Giovanni di Minno,4 and Francesco Salvatore1 (1 Dipartimento di Biochimica e Biotechnologie Mediche, Università di Napoli “Federico II” and CEINGE-Biotechnologie Avanzate, I-80131 Napoli, Italy; 2 Facoltà di Scienze, Università del Molise, 86100 Isernia, Italy; 3 Centro Emofilia e Trombosi, Ospedale S.G. Bosco, 80141 Napoli, Italy; 4 Centro di Coordinamento Regionale Emocoagulopatie, Dipartimento di Medicina Clinica e Sperimentale, Università di Napoli “Federico II”, Napoli, Italy; * address correspondence to this author at: Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli “Federico II”, via S. Pansini 5, I-80131 Naples, Italy; fax 39-081-746-3650, e-mail salvator@unina.it)

Hemophilia B (HB) is an X-linked recessive bleeding disorder caused by mutations that produce factor IX (FIX)
deficiency. The incidence of HB is ~1:30 000 live male births. The FIX gene spans ~34 kb and contains eight exons. The disease results from a myriad of mutations, and because of the rapid turnover of FIX mutations, there is no common mutation pattern in any ethnic group (1). Carrier and prenatal diagnosis can be made by linkage analysis (1, 2), which is rapid and inexpensive but limited by noninformative families, recombinant events, and the high incidence of germline mutations (1). Denaturing gradient gel electrophoresis (3, 4) and direct gene sequencing (5–8) have been used for the direct identification of FIX mutations. Denaturing reversed-phase HPLC (D-HPLC), which has been used to scan several disease genes, is more sensitive than other scanning procedures and less expensive than direct sequencing (9–13). In addition, the post-PCR analysis can be automated.

We developed an original D-HPLC screening procedure for the whole FIX gene and analyzed a cohort of 18 unrelated HB patients from Southern Italy previously typed by direct sequencing. In all patients, diagnosis was confirmed by FIX assay (Table 1). The study was approved by the institutional ethics committee.

After obtaining informed consent, we collected blood samples by venipuncture at the time of sampling for routine molecular analyses and extracted the DNA with the “Nucleon” procedure (Amersham). Each DNA sample was amplified by PCR for all FIX exons and for the promoter region and then was analyzed by sequencing and by D-HPLC. FIX exons 2 to 7 were amplified using primers described elsewhere (5). Exon 1 and the upstream region of FIX (i.e., from the nucleotide –482), which includes the promoter, were amplified using novel primers:

Promoter forward: 5′-TCTCCCTCAATGGGTCTTTG-3′
Exon 1 forward: 5′-TTCAGACTCAATACCCACA-3′
Exon 1 reverse: 5′-AAAAGGCAAGCATCTAATGT-3′

Exon 8 was amplified in two fragments of ~400 bp, each using the following novel primers:

Exon 8 (proximal) forward: 5′-TTGCAGATTAGGTCAGTGGTC-3′
Exon 8 (proximal) reverse: 5′-ATGTGGCTGCGTCAACAGTACTG-3′
Exon 8 (distal) forward: 5′-TTTCAGCAATTACGAAAC-3′
Exon 8 (distal) reverse: 5′-GCTCTGTTAATTTTCAATTCCA-3′

The PCR mixture included, for each amplification, the following reagents in a final volume of 50 μL: 100 ng of DNA, 1× PCR buffer (Applied Biosystems), 250 μM each deoxynucleotide triphosphate (Amersham), 600 nM each of the primers (forward and reverse), 1.5 mM MgCl2, and 1 U of Taq polymerase (Applied Biosystems). The PCR conditions were set up to amplify all of the fragments with the same program. The protocol was as follows: denaturation, 1 cycle at 94 °C for 3 min; amplification, 14 touchdown cycles at 94 °C for 20 s, 61 °C for 40 s (decreasing 0.5 °C/cycle), and 72 °C for 45 s; 25 cycles at 94 °C for 20 s, 54 °C for 40 s, and 72 °C for 45 s; and primer extension, 1 cycle at 72 °C for 7 min.

Sequence analysis was performed using the Sanger protocol (14) with an automated procedure in which the four terminator reactions were marked with fluorescent dideoxynucleotides; the fragments were analyzed with the 3100 genetic analyzer (Applied Biosystems). We used the WAVE system 3500 (Transgenomic) for

<table>
<thead>
<tr>
<th>Patient</th>
<th>FIX concentration, %</th>
<th>Exon</th>
<th>Optimum temperature, °C</th>
<th>Initial concentration buffer B, %</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
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<tr>
<td>1</td>
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<td>57</td>
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<td>6488C→T</td>
<td>T38I</td>
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<td>58</td>
<td>52</td>
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<td>C95R</td>
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<td>52</td>
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<tr>
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D-HPLC analysis. PCR samples from HB patients were mixed with a PCR sample from a healthy individual, denatured at 95 °C for 5 min, and left at room temperature for 45 min, a procedure that allowed heteroduplexes to form if a mutation was present in the sample from the patient. DNA aliquots (5–8 μL) were loaded on a preheated C\textsubscript{18} reversed-phase column [DNASep; 4.5 (i.d.) × 50 mm; Transgenomic]. The oven temperature for partial heteroduplex separation with partial DNA denaturation was deduced from the melting profile of the DNA sequence. Wavemaker 4.1.40 software (Transgenomic) was used to compute melting curves and to establish the temperature for analysis, i.e., the temperature at which 30% of the sequence was denatured. DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mmol/L triethylamine acetate (TEAA) buffer, pH 7.0, (Transgenomic) at a constant flow rate of 0.9 mL/min. The gradient was formed by mixing buffer A (0.1 mmol/L TEAA) and buffer B (0.1 mmol/L TEAA containing 250 mL/L acetonitrile). The analytic gradient lasted 4 min, and buffer B was increased by 2% per min. For each fragment, the initial concentration of buffer B was adjusted to obtain a retention time of 4–5 min (see Table 1). The column was then cleaned with 100% buffer B for 30 s and equilibrated at the starting conditions for 6 s before the next injection. Elution of DNA was detected by the absorbance at 260 nm. HSM software (Transgenomic) regulated each setting of the Wave system during analysis and stored the data.

Sequence analysis identified a mutation in all 18 DNA samples from our HB patients (Table 1). All variants were point mutations: 4 were nonsense, and the other 14 were missense mutations. A gene variant (−323T→C) was identified 323 nucleotides upstream from the gene in a patient who also carried the A233T mutation in exon 7 (Table 1, case 9). These findings confirm the heterogeneity of FIX mutations (1, 15). In fact, unlike other frequent genetic disorders, such as hemophilia A, in which ∼50% of patients carry an inversion of F8C intron 8 (1), and cystic fibrosis, in which the microdeletion DF508 is present in up to 80% of mutated alleles (16), no predominant or ethnicity-specific mutations were present in the FIX gene in patients with HB.

The D-HPLC method identified all 18 mutations. We first performed D-HPLC using the temperature indicated by the Wavemaker software; we then modified the run temperatures in 1 °C steps. For most fragments, the optimal temperature (shown in Table 1) exceeded that indicated by the software by 1–4 °C; in fact, an increase in oven temperature caused a decrease of the retention time, thereby allowing better resolution of the heteroduplexes.

Several examples of D-HPLC profiles obtained in patients bearing FIX mutations as compared with the D-HPLC wild-type profile are shown in Fig. 1. Fig. 1A shows a DNA sample bearing the T38I mutation in exon 2 compared with the wild-type profile; the mutation appears as a two-peak profile. Fig. 1B shows a DNA sample bearing the A148T polymorphism (exon 6); in this case, the DNA sample bearing the variant also appears as a two-peak profile. Fig. 1C shows a DNA sample bearing a double variant of exon 6, i.e., the A148T polymorphism and the R180W mutation. In this case there were three peaks.

The D-HPLC procedure is reproducible: all 18 samples from HB patients were analyzed twice by two operators who obtained the same results. In addition, our study confirms the high sensitivity of D-HPLC reported in the evaluation of CFTR mutations (9, 10) and several other genes (13). The sensitivity of D-HPLC depends on three factors: (a) Each DNA sample must be run at its optimal temperature. A large spectrum of run temperatures must be used with large DNA fragments and with DNA fragments that have several melting domains with different temperatures (9); several software packages are available to calculate the optimal D-HPLC conditions [see, e.g., the Stanford web site (http://insertion.stanford.edu/melt.html)]. (b) The formation of heteroduplexes in DNA
samples bearing mutations must be correct. In the case of X-linked diseases, this requires the equimolar mixing of PCR products between a wild-type DNA sample and the sample to be tested. (c) The type of mutations searched for must be detectable by D-HPLC. D-HPLC is more sensitive in the screening of point mutations, which is the case of most FIX mutations (1). In any case, once the D-HPLC conditions have been defined, a prospective study of a novel population sample should be performed to confirm the detection rate of the D-HPLC procedure.

The D-HPLC scanning procedure described here is fast because all of the DNA fragments can be amplified under the same conditions, the post-PCR phase is automated, and each D-HPLC run requires only 6 min. The protocol for D-HPLC scanning of the entire FIX gene is completed in <5 h. Finally, the D-HPLC method is cost-effective: we calculated that it costs approximately US $25 to scan the whole FIX gene, excluding instrument and personnel costs. Direct sequencing has a 100% sensitivity, and rapid protocols have been set up to analyze all FIX gene fragments under the same PCR conditions (6) or in single multiplex PCR amplifications (5). Direct FIX gene sequencing has been shown to be efficient in various ethnic-geographic groups (5–8). However, direct sequencing is more expensive than D-HPLC. On the other hand, scanning procedures have a sensitivity of ~75–90% (10), and in most studies D-HPLC was more sensitive than other scanning procedures, as recently demonstrated for the factor VIII gene (11) and for several other disease genes (12, 13). Furthermore, denaturing gradient gel electrophoresis and single-strand conformation polymorphism analysis are more difficult to automate (9).

In conclusion, D-HPLC scanning of the FIX gene with the procedure described here is suitable for the routine diagnosis of HB and for carrier diagnosis if the proband is not available. Using this procedure, we have confirmed the heterogeneity of FIX gene mutations in HB patients from Southern Italy.

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References


Fasting vs Nonfasting Plasma Homocysteine Concentrations for Diagnosis of Hyperhomocysteinemia, M. Rebecca Fokkema1*, Marleen F. Gilissen,1 Jasper J. van Doormal,2 Marcel Volmer,1 Ido P. Kema,1 and Frits A.J. Muskiet1


Fasting vs Nonfasting Plasma Homocysteine Concentrations for Diagnosis of Hyperhomocysteinemia, M. Rebecca Fokkema1*, Marleen F. Gilissen,1 Jasper J. van Doormal,2 Marcel Volmer,1 Ido P. Kema,1 and Frits A.J. Muskiet1 (Departments of 1Pathology and Laboratory Medicine and 2Internal Medicine, University Hospital Groningen, NL-9700 RB Groningen, The Netherlands; * address correspondence to this author at: Pathology and Laboratory Medicine, University Hospital Groningen, CMC-V, Room Y1.165, PO Box 30.001, NL-9700 RB Groningen, The Netherlands; fax 31-50-3612290, e-mail m.r.fokkema@path.azg.nl)

Hyperhomocysteinemia, a risk factor for cardiovascular and thrombotic disease, pregnancy complications, and cognitive disorders, is defined as a fasting plasma total Hcy (tHcy) above a chosen cutoff value (1, 2). Reducing both the analytical and biological variation may add to the diagnostic value of any test. tHcy analytical variation (CVt) is method-dependent and ranges from 2.7% to 4.9% for fluorescence polarization immunoassays and from 2.5% to 14% for HPLC assays (3). The reported biological variations for tHcy under fasting or otherwise standardized conditions include 20–34% interindividual variation (CVi) and 7–11% (with a single extreme of 15–17%) intra-individual variation (CVi) (4–9). Because of the short-term influence of meals, e.g., protein, on CVi (10), it is generally recommended that blood samples be collected under fasting or otherwise meal-standardized conditions. The influence of protein intake on between-day CVi is, however, unknown. The necessity for fasting has