likely the cause of the EPP condition in the patient. Because the patient is a carrier of a missense mutation rather than a null-allele mutation, she is highly unlikely to develop EPP-related liver complications later in life, based on current knowledge (3).

The other three members of the family were screened specifically for the L182R mutation by DGGE. As shown in Fig. 1A, T545→G (L182R) was also present in the DNA sample from proband’s father but was absent in samples from both her mother and her brother. The molecular analysis confirmed that the proband’s father was an asymptomatic carrier of mutation L182R.

As discussed earlier, two SNPs in the ferrochelatase gene, namely, −251A/G in the promoter region and −23C/T in intron 1, play a role in the pathogenesis of EPP (4). To verify the latest findings, the two SNPs were studied in all four members of the family, using the method described by Gouya et al. (4). Both the proband and her mother were heterozygous for both SNPs, whereas the proband’s father and brother were homozygous (−251A; IVS1-23C; Fig. 1B). Mutation L182R resided in one of the (−251A; IVS1-23C) alleles in the father, which was passed to his daughter, the proband. From her mother, the proband inherited a wild-type FECH allele featuring the haplotype (−251G; IVS1-23T). The mutation-bearing allele features the haplotype (−251A; IVS1-23C) and is indicated by the boxed AC. The allele that is responsible for the clinical manifestation of EPP featuring the haplotype (−251G; IVS1-23T) is indicated by the bold italic GT.

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References

Measurement of Cholesterol and Triglycerides in Dried Serum and the Effect of Storage, Lakshmy Ramakrishnan, K. Srinath Reddy, and Bansi Lal Jailkhani (Departments of 1 Cardiology and 2 Laboratory Medicine, All India Institute of Medical Sciences, N. Delhi-110029, India; * address correspondence to this author at: Department of Cardiology, CNC, All India Institute of Medical Sciences, N. Delhi-110029, India; fax 91-11-6862663, e-mail lakshmy_ram@yahoo.com)

Dried blood on filter paper has been found to be suitable in large-scale population screening programs (1–3), but to the best of our knowledge, dried blood or serum on filter paper has not been used for lipid measurements. In the present study, we evaluated the stability of cholesterol and triglycerides in serum dried on filter paper.

Fifty-four patients coming to the centralized laboratory, CNC, All India Institute of Medical Sciences for lipid investigations were selected at random, and blood was collected by venipuncture into tubes without anticoagulant. Serum was separated within 2 h of collection. All
procedures were in accordance with the ethics standards of our institution. An aliquot of each serum sample was analyzed immediately. Exact 10-μL replicates of the samples were also spotted onto 3M Whatman filter paper kept on a nonabsorbent surface (thermacol) and left at room temperature for 1 h for drying. The room temperature was 16–37 °C for the duration of the study.

After drying, one aliquot was eluted and analyzed on the day of collection. The remaining filter discs were kept in a sealed plastic bag to protect them from dust and moisture and stored at room temperature for different time periods. At 7, 14, 21, 28, and 35 days, entire dried serum spots corresponding to 10 μL were cut out with scissors and transferred to 1.0 mL of enzymatic reagent (described below). For fresh serum, 10 μL was added to 1 mL of the reagent. The enzymatic reagent for cholesterol contained cholesterol esterase, cholesterol oxidase, peroxidase, phenol, and 4-amino antipyrine in PIPES buffer and was purchased from RANDOX. For fresh serum, the reaction was carried out at room temperature for 10 min; for dried serum on filter paper, the reaction time was 30 min. (We observed that 10 min was not sufficient for the reaction to reach completion.) The absorbance of the resulting product was measured at 500 nm on a Spectronic 20 spectrophotometer. Calibration was with calibrators dried on filter discs and treated the same way as samples. For triglyceride measurements in fresh serum (10 μL) or on filter discs, the enzymatic reagent contained lipase, glycerol kinase, glycerol-3-phosphate oxidase, peroxidase, ATP, 4-chlorophenol, and 4-amino antipyrine in PIPES buffer.

The interassay CV of the modified method for filter discs was 2.6% for cholesterol and 2.4% for triglycerides; these compared well with the original method (2.8% and 2.5%, respectively). For quality assurance, the laboratory is part of United Kingdom National External Quality Assessment Scheme (UKNEQAS) program for cholesterol and triglycerides. Statistical analysis was done by within-subject ANOVA.

Both cholesterol and triglycerides were completely released from the filter discs in the respective enzymatic reagents within 30 min (calibrators dried on filter discs gave 100% recovery). Total cholesterol values in the 54 samples (fresh) were 2.57–7.05 mmol/L. Forty-one samples had cholesterol values ≤5.2 mmol/L, and 13 samples had values >5.2 mmol/L. The mean recovery of cholesterol from dried sera was 99.4–99.8% at different time points.

Triglyceride values in the 54 samples were 0.736–6.854 mmol/L. Triglyceride values were ≤1.725 mmol/L in 22 samples and >1.725 mmol/L in 32 samples. One subject had a triglyceride concentration of 6.854 mmol/L. The mean recovery of triglycerides from dried serum at different time points was 100.6–101.7%.

The mean cholesterol and triglyceride concentrations in fresh samples and the dried serum samples at the end of the different storage times are shown in Table 1. Mean cholesterol in dried serum eluted and measured on the day of sample collection (day 0) did not differ signifi-

<table>
<thead>
<tr>
<th>Days of storage</th>
<th>Cholesterol, mmol/L</th>
<th>Triglycerides, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 (liquid serum)</td>
<td>4.575 ± 1.103</td>
<td>1.967 ± 1.014</td>
</tr>
<tr>
<td>Day 0 (dried serum)</td>
<td>4.562 ± 1.105</td>
<td>1.975 ± 1.023</td>
</tr>
<tr>
<td>Day 7</td>
<td>4.523 ± 1.066</td>
<td>1.974 ± 1.000</td>
</tr>
<tr>
<td>Day 14</td>
<td>4.535 ± 1.040</td>
<td>1.971 ± 1.016</td>
</tr>
<tr>
<td>Day 21</td>
<td>4.544 ± 1.041</td>
<td>1.970 ± 1.001</td>
</tr>
<tr>
<td>Day 28</td>
<td>4.545 ± 1.048</td>
<td>1.975 ± 1.000</td>
</tr>
<tr>
<td>Day 35</td>
<td>4.536 ± 1.052</td>
<td>1.984 ± 1.024</td>
</tr>
</tbody>
</table>

Repeated measures (ANOVA) F = 0.97 F = 0.89
P = 0.45 P = 0.50

*Values are mean ± SD of 54 samples. Cholesterol and triglycerides were estimated in the fresh serum (liquid) and the dried serum at different time periods as described in the text. The values obtained at different time periods were compared by within-subject ANOVA.

Fig. 1. Regression curves for cholesterol (A) and triglycerides (B) measured in fresh serum and serum dried on filter paper. Cholesterol and triglyceride values (n = 54) obtained in serum dried on filter discs and measured on day 0 were correlated with the values obtained in fresh serum (liquid). Equations for the lines: (A), y = 0.997x (r² = 0.978); (B), y = 0.995x (r² = 0.996).
filter discs at room temperature for 7, 14, 21, 28, and 35 days also did not significantly alter the mean cholesterol values, as is apparent from the $F$ statistic (0.97) and the $P$ value (0.45; Table 1).

The mean triglyceride concentrations given in Table 1 indicate that dried serum is stable for triglyceride measurements and that storage for different time periods at room temperature does not alter the mean values significantly ($F = 0.89; P = 0.50$). An excellent correlation was observed between triglyceride concentrations in fresh samples and samples dried on filter paper and analyzed on day 0 (Fig. 1B).

Dried blood on filter paper may be useful for cholesterol and triglycerides. It has been used extensively for mass screening programs (1, 2, 6–8) and for measurement of glucose, insulin, glycosylated hemoglobin (9, 10), and steroids (11).

The adaptation of lipid assays to dried blood is ideal for pediatric applications and in multicenter studies where the costs and safety of sample transportation to a distant laboratory are limiting considerations. However, because serum was used in the present study, this approach needs to be evaluated in whole blood, and the use of filter paper matrix as a means of sample transportation would also need to be validated in the context of a multicenter study.

We conclude that cholesterol and triglycerides are highly stable in dried serum and are readily transferable to a liquid phase. The good agreement between values in dried serum and fresh samples supports the validity of the measurement of these analytes in dried serum.

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References


Mutation Screening of the Entire Coding Region of the Protoporphyrin Oxidase Gene Using Denaturing Gradient Gel Electrophoresis and Denaturing HPLC, Lene Christiansen,1 Anette Bygum,2 Marianne Kæhne,1 Alice Jensen,1 Magnes Harder,1 and Niels Erik Petersen1 (1 Department of Clinical Biochemistry and Clinical Genetics, Odense University Hospital, DK-5000 Odense C, Denmark; 2 Department of Dermatology, Martselsborg Hospital, 8000 Aarhus, Denmark; *author for correspondence: fax 45-6541-1911, e-mail lene.christiansen@ouh.fyns-amt.dk)

Variegate porphyria (VP) is characterized by decreased activity of protoporphyrinogen oxidase (PPOX), the penultimate enzyme in the heme biosynthetic pathway. VP belongs to the group of mixed porphyrias and presents with both cutaneous manifestations and acute neurovisceral attacks. Genetically, VP is associated with mutations in the gene encoding PPOX and is usually inherited as an autosomal dominant trait with low clinical penetrance (1, 2). Although a few founder mutations predominate in certain populations, VP generally is a hetroallelic disease, with a variety of mutations in the PPOX gene being responsible for the decreased activity of PPOX (3, 4). The 5.5-kb PPOX gene is located on chromosome 1q22-23 and contains 1 noncoding and 12 coding exons (5). To date, >80 different mutations and polymorphisms have been identified in the PPOX gene (3–12).

Characterization by mutational analysis of suspected cases of VP should be considered an important clinical diagnostic tool because of the similarities in clinical presentation of VP, porphyria cutanea tarda (PCT), and hereditary coproporphyria. Furthermore, the use of DNA diagnostic methods enables detection of latent or asymptomatic mutation carriers at risk of developing VP.

Here we describe assays for easy and reliable screening of the entire coding region of the PPOX gene as well as 74 bp of the noncoding exon 1 and 8–41 bp of the flanking intron sequences, using denaturing gradient gel electrophoresis (DGGE) and denaturing HPLC (dHPLC). Because VP frequently is confused with the more common PCT, we screened for PPOX mutations in DNA from 40 sporadic PCT patients, using established assays to detect possible misclassified VP cases.

The study protocol was approved by the relevant Regional Ethical Committees. All participants received written information and gave signed consent to participate in the study.

The 40 patients included in the study were previously diagnosed as having PCT based on the clinical presentation and the urine porphyrin excretion patterns. Feces porphyrin measurements were not available. Genetic analysis for disease-related mutations in the uroporphyrinogen decarboxylase gene were negative (13).

DNA was isolated from 10 mL of EDTA-stabilized blood by standard procedures (14).

The primers used for PCR amplification of 13 DNA fragments covering the 3′ part of the noncoding exon 1

$F = 0.89; P = 0.50$; $R^2 = 0.97$.