Development and Validation of an Enzymatic Method for Total Cholesterol Analysis Using Whole Blood Spot

Gaetano Corso,^{1*} Francesco Papagni,^{2,‡} Monica Gelzo,^{1,2,‡} Monica Gallo,² Rosalba Barone,² Maria Graf,² Nicola Scarpato,² and Antonio Dello Russo^{2*}

¹Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy

²Department of Molecular Medicine and Medical Biotechnologies, University of Naples Federico II, Naples, Italy

Background: High serum cholesterol represents a risk factor for cardiovascular disease. This study aims to quantify total cholesterol in dried blood spot (DBS) by direct enzymatic method. **Methods:** Three hundred seventeen blood samples with serum cholesterol level ranging from 81 to 337 mg/dl were collected. DBS were manually prepared, cholesterol was extracted using methanol and analyzed by a manual enzymatic method. DBS cholesterol method was validated for imprecision and extraction efficacy. DBS cholesterol values were correlated (training test) with serum values measured by automated enzymatic method (reference method). The obtained correlation was used for predicting serum cholesterol from DBS analysis of a new sample group (validation test, $n = 58$). **Results:** Within-day and between-day coefficient of

variation (CV%) were lower than 7.69 and 6.32, respectively. Residual cholesterol in DBS after extraction was 16%. DBS cholesterol and serum cholesterol showed a linear correlation (slope = 0.5217; $r = 0.9139$) and a bias of -28% . Furthermore, DBS cholesterol values of validation test ($n = 58$), converted using the training test correlation, were not statistically different compared to the corresponding plasma values ($P = 0.9487$), and the comparison by Passing and Bablok showed a linear regression with a slope of 1.068 ($r = 0.611$) and a bias of -0.22% . **Conclusions:** The results show that this enzymatic method is suitable to analyze cholesterol in DBS and it could be automated and used for population screening of total blood cholesterol. J. Clin. Lab. Anal.

30:517–523, 2016. © 2015 Wiley Periodicals, Inc.

Key words: total blood cholesterol; dried blood spot; chronic degenerative diseases

INTRODUCTION

From the 1950s to date, population aging in developed and developing countries has been observed. This led to epidemiological transition by addressing public health concerns on chronic degenerative diseases, such as cardiovascular disease (CVD), cancer, and diabetes. In 2008, as reported by the World Health Organization (WHO), approximately 17.3 million of people died from CVD accounting for 30% of all global deaths. These include atherosclerosis, and its complications still occupy the first place among the causes of mortality and morbidity in Western countries (1). The prevalence of atherosclerosis is constantly increasing also in nonindustrialized countries, and it is estimated that in 2020 the diseases depending on atherosclerosis will represent a worldwide leading cause of mortality (2). Atherosclerosis is conventionally defined as a chronic systemic inflam-

matory disease, which can remain undetected for many years before the onset of clinical symptoms represented mainly by coronary artery disease, cerebral ischemia, and peripheral arterial disease (3–5). High levels of cholesterol and triglycerides have been recognized as important risk factors for CVDs (6, 7). The blood contains

[‡]These authors shared the position.

*Correspondence to: Gaetano Corso, Department of Clinical and Experimental Medicine, University of Foggia, Viale L. Pinto, 1, 71122 Foggia, Italy. E-mail: gaetano.corso@unifg.it
Antonio Dello Russo, Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, via Pansini, 5, 80131 Naples, Italy. E-mail: antonio.dellorusso@unina.it

Received 19 August 2014; Accepted 10 August 2015

DOI 10.1002/jcla.21890

Published online in Wiley Online Library (wileyonlinelibrary.com).

about 0.18% of cholesterol present in free form mostly in erythrocyte membranes, while in plasma it occurs both in free form (20–30%) and esterified to fatty acids (60–70%), and it is carried by lipoproteins from one tissue to another (8).

Total serum cholesterol is routinely analyzed by enzymatic colorimetric methods (9, 10), but in many developing countries, biochemical analysis of total cholesterol is very difficult due to the lack of resources and technical expertise, and the analysis performed on dried blood spot (DBS) may be a viable alternative. DBS is obtained by collecting a drop of capillary blood on filter paper by heel or finger prick (11). Compared to the analysis of plasma/serum, the analysis of DBS offers several advantages, such as reduction of invasiveness of blood sampling, a smaller amount of sample, a reduction in costs, as well as a reduction of infection risk through the deactivation of potential pathogens, such as HIV (11–13). Methods for the measurement of cholesterol and triglycerides from DBS have already been reported in previous studies (14–17). In addition, it may be useful for population screening not only for cholesterol, but also for other lipids (18, 19). Whole blood cholesterol levels could be analyzed at home by some commercialized “cholesterol testing kit” using a fresh drop of blood, but the analysis is expensive and not useful for screening. In addition, the analytical performances are not yet well established by accurate studies.

This study was aimed to quantify the total cholesterol in DBS with an enzymatic method, to correlate DBS cholesterol to serum values obtained by an automated method routinely used in labs (reference method), and to predict serum cholesterol levels from DBS values. For this purpose, it has been developed and validated by a method for the extraction of total cholesterol from DBS samples, and its analysis by a manual enzymatic method. The method was used for analyzing samples from subjects with normal and pathological serum cholesterol levels, and the range of total cholesterol levels in DBS was reported.

MATERIALS AND METHODS

Reagents and Samples

Automated and manual enzymatic colorimetric method for cholesterol analysis was performed using CHOD/POD/TRINDER reagent and calibrator (Roche Diagnostics S.p.A., Milano, Italy). In this method, cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and free fatty acids. Cholesterol oxidase then catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide formed affects the oxidative coupled system of phenol and 4-aminoantipyrine to form a red quinoneimine

dye. The color intensity of the dye formed is directly proportional to the cholesterol concentration. It is determined by measuring the increase in absorbance. Pure solvents, methanol, dichloromethane, hexane, acetone, ethanol, and chloroform were purchased from JTBaker (Deventer, Netherlands). Potassium hydroxide (KOH) was purchased from Merck (Merck KGaA, Darmstadt, Germany). The internal standard for gas chromatography-flame ionization detector (GC-FID) analysis, 5- α -cholestane (Sigma, St. Louis, MO), was dissolved in a mixture of chloroform/methanol (2:1) to a final concentration of 0.4 mg/ml.

Blood samples (317 for training test and 58 for validation test), collected in EDTA tubes, with total serum cholesterol levels ranging between 81 and 337 mg/dl were selected from samples already analyzed in our labs (the group aged from 20 to 60 years). From each sample, five DBS were prepared spotting 70 μ l of whole blood on Whatman 903[®] filter paper cards (Whatman GmbH, Dassel, Germany). The DBS were left to dry at room temperature for 2–3 h, protected from light, and stored at 4°C until analysis (20).

Quality controls (QC) (DBS) with low and high cholesterol levels (low QC, $n = 50$; high QC, $n = 50$) were prepared, as described above, using two whole blood samples with serum cholesterol of 147 and 274 mg/dl, respectively.

DBS Cholesterol Analysis

Total cholesterol was extracted from a disk of 6 mm, punched from a DBS, corresponding to 12.4 μ l of whole blood. For the extraction, the disk was incubated with 100 μ l of methanol at 37°C for 2 h on a shaking plate. The determination of cholesterol was performed using 60 μ l of the extract mixed with 600 μ l of the reagent. The reaction mixture was incubated at 37°C for 15 min, and the absorbance at 505 nm was measured. For the calibration curve, 10 μ l of calibrator (or water for blank) and 50 μ l of methanol were used. Hence, DBS cholesterol values were corrected using a dilution factor of 1.344.

Method Validation

DBS cholesterol method was validated for imprecision and extraction efficacy. Furthermore, DBS values were correlated with serum cholesterol analyzed by reference method (automated enzymatic colorimetric method), and this correlation was used for predictive evaluation.

Within-day imprecision was evaluated analyzing five times each level of QC samples in the same analytical run. For evaluation of between-day imprecision, each QC sample was analyzed once a day for 31 days.

The evaluation of extraction efficiency was performed by a GC-FID method (21) on three different DBS samples with serum cholesterol levels of 135, 195, and

TABLE 1. Within- and Between-Day Imprecision Study of DBS Cholesterol Measured in the DBS Quality Controls at Two Concentration Levels

	Within-day (<i>n</i> = 5)		Between-day (<i>n</i> = 31)	
	Mean (mg/dl)	Imprecision (CV%)	Mean (mg/dl)	Imprecision (CV%)
Low QC	142	3.98	147	5.97
High QC	199	7.69	184	6.32

260 mg/dl (*n* = 15) and analyzed in quintuplicate. The residual cholesterol in DBS was evaluated after the extraction for 30, 60, and 120 min of incubation in methanol. Briefly, after the methanol extraction, the spot and 20 µg of 5- α -cholestane were mixed with 1 ml of 1N KOH in 90% ethanol and hydrolyzed at 80°C for 1 h. After cooling and addition of 1 ml of distilled water, the extraction of sterols was carried out with 2 ml of hexane for three times. The hexane was removed under a gentle stream of N₂, the dry residue was resuspended in 100 µl of dichloromethane, and 1 µl of solution was injected into GC apparatus. The residual cholesterol in DBS was expressed as percentage of total cholesterol in DBS measured by GC-FID standard method (21).

The DBS cholesterol values (*n* = 317) were correlated with serum cholesterol previously analyzed by automated enzymatic colorimetric method (reference method; Modular Analytics, Roche Diagnostics S.p.A.), and used as training test. The above regression curve was employed for predicting the serum cholesterol levels using DBS values measured in a different group of samples (*n* = 58; predictive calculation group). Thereafter, the cholesterol predicted values were compared with those obtained in the corresponding plasma samples by reference method (validation test).

Statistical Analysis

Data were reported as mean, standard deviation, and percentiles (%ile). Statistical differences were assessed by Student's *t*-test. Outliers were evaluated by Tukey fence (TF) method. The comparison between methods was per-

formed by Passing and Bablok regression, and Bland-Altman analysis.

RESULTS

Table 1 shows imprecision values of the enzymatic method evaluated on high and low DBS-QC analyzed during the assays of patient samples. Within-day imprecision (CV%) was calculated by analyzing each QC level five times in the same analytical run, and the results ranged from 3.98% to 7.69%. The between-day imprecision, calculated by analyzing each QC level once a day for 31 working days, ranged from 5.97% to 6.32%.

The results of cholesterol residue in DBS after the extraction step are reported in Table 2. The residual cholesterol, analyzed by GC-FID, in three different DBS samples after 30, 60, and 120 min of extraction times with methanol, decreased during the time of incubation, and after 120 min was on average 16%. The percentage of residual cholesterol was also used for correcting the DBS cholesterol values.

Table 3 reports the descriptive statistics of cholesterol values in serum and DBS analyzed by the reference-automated method and DBS enzymatic method, respectively. All data were elaborated by a robust statistic method to identify and reject the outliers below the low TF and above the high TF. The TF analysis for serum cholesterol showed a low TF of 24 mg/dl and a high TF of 368 mg/dl. For DBS cholesterol, a low TF of 83 mg/dl and a high TF of 252 mg/dl were found (Table 3). No data were excluded by TF analysis for cholesterol in serum and in DBS.

TABLE 2. Evaluation of Residual Cholesterol in DBS by GC-FID Method After Different Extraction Times With Methanol

Samples (<i>n</i>)	Total cholesterol in DBS (mg/dl)	Residual cholesterol in DBS (30', %) ^a	Residual cholesterol in DBS (60', %) ^a	Residual cholesterol in DBS (120', %) ^a
	Mean (SD)			
Low (5)	116 (9)	21 (6)	18 (4)	15 (6)
Medium (5)	152 (11)	22 (7)	18 (5)	16 (3)
High (5)	179 (13)	22 (6)	21 (2)	18 (5)

^aTime of extraction with methanol and percentage of residual compared to total cholesterol.

TABLE 3. Comparison Between Values Obtained by Reference Method in Serum and by Manual Enzymatic Method in DBS

	Serum (mg/dl)	DBS (mg/dl)
<i>n</i>	317	317
Mean (SD)	195 (57)	167 (31)*
Mean ± 2SD	81–309	105–229
Median	192	166
1° quart	153	146
3° quart	239	188
IQR	86	42
TF low	24	83
TF high	368	252
Min–max	81–337	103–251
2.5°–97.5° %ile	94–296	113–228
g1/s1	0.67	1.21
g2/s2	–2.53	–1.68

*Statistically significant ($P < 0.0001$). IQR: Interquartile Range.

The distribution of cholesterol concentrations obtained in serum and DBS, compared to theoretical Gaussian distribution, was expressed using the coefficient of asymmetry (g1) and kurtosis (g2), which indicate the type of deviation from normality. Coefficients (g1) and (g2) are considered significant if they exceed 2.6 times the standard deviations (s1) and (s2), respectively. As reported in Table 3, ratios of the coefficient of asymmetry (g1/s1) were 0.67 for serum cholesterol and 1.21 for DBS cholesterol, and ratios of the coefficient of kurtosis (g2/s2) were –2.53 for serum cholesterol and –1.68 for DBS cholesterol. These results show that cholesterol distributions in both serum and DBS do not differ statistically from a normal Gaussian distribution.

Serum cholesterol values calculated by parametric method (mean ± 2SD) ranged from 81 to 309 mg/dl and included more than 98% of total samples with only six values above the upper limit. DBS cholesterol values ranged from 105 to 229 mg/dl and included the 96.8% of values, with only two values below the lower limit and eight values above the upper limit. However, DBS cholesterol values show a concentration range narrower (about 45%) than serum cholesterol values. The average of DBS cholesterol (167 ± 31 mg/dl) was significantly lower of about 14% than serum cholesterol (195 ± 57 mg/dl; $P < 0.0001$), and in agreement with other studies (22, 23).

In addition, the Passing and Bablok regression study was performed between DBS values and those obtained in sera ($n = 317$), and this regression has been used as training test. As depicted in Figure 1A, the comparison between the two groups of values showed a linear correlation ($y = 0.5217x + 64.86$; $r = 0.9139$). Furthermore, the regression study was also performed on data divided by gender (156 males and 161 females). As reported in Figure 1(B and C), both regressions also showed linear correlations ($y = 0.5353x + 63.13$; $r = 0.914$ for males,

Fig. 1. Passing–Bablok regression analysis of DBS cholesterol levels and serum cholesterol levels in healthy subjects (training test). (A) All subjects, $n = 317$. (B) Males, $n = 156$. (C) Females, $n = 161$. The x -axis represents the measurements by the reference method (automated enzymatic method) and the y -axis the manual enzymatic method (DBS). The diagonal line represents the line of identity, the heavier line shows the Passing–Bablok regression line, and the outer dotted lines show the allowable difference lines (20%).

Fig. 2. Passing–Bablok regression analysis of predicted serum cholesterol value from DBS (manual enzymatic method) and plasma cholesterol concentrations (reference method) in healthy subjects (validation test, $n = 58$). The predicted cholesterol value was obtained by conversion of DBS cholesterol value using the regression equation of training test. The x -axis represents the measurements by the reference method (automated enzymatic method), and the y -axis the manual enzymatic method. The diagonal line represents the line of identity, the heavier line shows the Passing–Bablok regression line, and the outer dotted lines show the allowable difference lines (20%).

and $y = 0.5082x + 66.51$; $r = 0.915$ for females). In addition, the DBS cholesterol differences observed between males and females were not statistically significant ($P = 0.285$).

A comparison has been also performed by Bland–Altman plot, which showed that most of the values obtained were within the confidence interval (95%) with only six points above the upper limit (<1.9%; 6/317) and seven points below the lower limit (2.2%; 7/317). The bias was equal to -28% , and a negative trend was observed from low to high levels of cholesterol, with an overestimation of DBS samples with lower cholesterol levels and an underestimation of DBS samples with higher cholesterol levels (plot not shown).

Furthermore, a validation test was performed using 58 new samples in which DBS cholesterol concentration was measured by manual enzymatic method; thereafter, the values were converted using the predictive equation of training test. Then, these values were compared to those measured by reference method in the corresponding plasma. As depicted in Figure 2, the Passing and Bablok comparison between predicted values obtained from DBS and plasma cholesterol showed a linear regression of $y = 1.068x - 17.6$ ($r = 0.611$), and the averages of two groups

Fig. 3. Bland–Altman plot between predicted serum cholesterol value from DBS (manual enzymatic method) and plasma cholesterol concentrations (reference method) in healthy subjects (validation test, $n = 58$).

were not statistically different ($P = 0.9487$). The Bland–Altman plot of validation test, showed in Figure 3, suggests that differences between cholesterol levels predicted from DBS analysis and cholesterol concentrations measured in plasma were within the confidence interval (95%) with only two points above the upper limit (<3.5%) and the bias was of -0.22% .

In addition, using a cutoff value of 190 mg/dl (24), we observed that 31 of 38 (81.6%) cholesterol values of validation test were below 190 mg/dl and in accordance with the corresponding plasma cholesterol values, and 11 of 20 (55.0%) were above 190 mg/dl and in accordance with the plasma values, instead discordant values were 7/38 and 9/20, respectively.

DISCUSSION

In this work, we developed and validated a method for the extraction and analysis of total cholesterol from whole blood adsorbed on paper (DBS). The analysis in DBS allows to reduce the degree of invasiveness of blood sampling, requires a small amount of sample, and the DBS can be easily sent to laboratories. Several markers are today analyzed in the blood spot to study populations for diagnostic and epidemiological purposes, and not least for drug monitoring (21, 25–31).

DBS cholesterol level represents the total amount of this metabolite in both plasma and erythrocyte membranes, and it could be clinically more informative than serum/plasma levels. In fact, a significant independent association between cholesterol of erythrocyte membranes in circulating red cells and clinical presentation of coronary artery diseases was recently described (32).

The method imprecision as CV% was less than 8% and the extraction procedure recovery was 84%. The

analytical variability of the method is acceptable, even though all DBS were extracted and analyzed manually. In addition, both analysis and extraction of cholesterol depend significantly on the quality of spot preparation. In fact, it is necessary to use filter paper calibrated for DBS analysis and the amount of blood to be deposited must be more or at least of 70 μ l, otherwise the values could be too variable. Also, it has been demonstrated that the stability of cholesterol in DBS can be longer than 2 months when stored at 4°C and in appropriate dehumidified containers (17).

The percentage of cholesterol residue in DBS after the extraction step was of 16%, and it was taken into account to obtain the accurate concentration of cholesterol in DBS. In this article, cholesterol was extracted from 317 DBS and analyzed in order to define DBS range of values, and to correlate DBS cholesterol with that of serum/plasma. The results obtained show that both correlation coefficient of training test ($r = 0.914$) and slope (0.5217) were in accordance with that reported by others (17, 22). The intercept was of 64 mg/dl, corresponding to about 20 mg/dl higher than those reported in the literature (17, 22). Therefore, DBS cholesterol levels were overestimated in samples with lower levels of cholesterol, on the contrary the samples with higher levels of cholesterol were underestimated. This trend can be explained if we consider that cholesterol content is stable in the erythrocyte membranes and on average its concentration is less variable compared to that found in serum/plasma. The amount of erythrocyte membrane cholesterol determines the negative trend observed in the Bland–Altman plot of training data set (plot not shown). This effect is well known considering that lipids in erythrocyte membranes result from an exchange between erythrocyte and circulating lipoproteins (33). In addition, it has been demonstrated that circulating cholesterol levels (serum/plasma) did not correlate with total cholesterol content of erythrocyte membranes probably because the latter represents the equilibrium achieved between red cell cholesterol influx and efflux, and intracellular cholesterol transport (32). Furthermore, even though the value of hematocrit in males is on average higher than that found in females, and thus it could influence the DBS cholesterol levels between males and females, we did not observe statistically significant differences of DBS cholesterol levels between genders.

The parameters of regression curve for cholesterol, obtained from training test between DBS and serum, have been used for predictive calculation of serum cholesterol levels using DBS values of unknown samples (validation test, $n = 58$). The predicted cholesterol levels, obtained from transformation of DBS values, showed a good correlation with cholesterol analyzed by reference method in the corresponding plasma samples. The correlation coef-

ficient (r) was 0.611, the slope was 1.068, and the intercept was -17.6 mg/dl. Moreover, differences between the predicted cholesterol from DBS and plasma cholesterol values in the 58 samples were not statistically significant ($P = 0.949$).

In conclusion, although the sample size of predictive group should be increased, this work describes preliminarily the equation for predictive calculation of serum cholesterol by using DBS cholesterol analysis. The method presented here can be easily automated, and used as routine analysis. Hence, it could be employed for population screening of total cholesterol directly from whole blood, circumventing the need for blood processing, storage, and shipment at low temperatures.

ACKNOWLEDGMENT

The authors are grateful to colleagues Dr. Antonio Del Rio, Dr. Concetta Sica, and Antonio Boscia (Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, of Naples) for their valuable collaboration.

REFERENCES

1. Jemal A, Ward E, Hao Y, Thun M. Trends in the leading causes of death in the United States, 1970–2002. *JAMA* 2005;294:1255–1259.
2. Murray CJL, Lopez LD. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *Lancet* 1997;349:1498–1504.
3. Viles-Gonzalez JF, Fuster V, Badimon JJ. Atherothrombosis: A widespread disease with unpredictable and life-threatening consequences. *Eur Heart J* 2004;25:1197–1207.
4. Li JJ, Fang CH. Atherosclerosis is a more rational term for the pathological entity currently known as atherosclerosis. *Med Hypotheses* 2004;63:100–102.
5. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation* 2005;111:3481–3488.
6. Sprecher DL. Triglycerides as a risk factor for coronary artery disease. *Am J Cardiol* 1998;82:49U–59U.
7. Castelli WP. Cholesterol and lipids in the risk of coronary artery disease—the Framingham Heart Study. *Can J Cardiol* 1988;4(Suppl A):5A–10A.
8. Chatterjea MN, Shinde R. 2012. Chemistry of lipids. In: R Saxena, editor. *Textbook of Medical Biochemistry*. New Delhi: JP Medical Ltd.; p 45–65.
9. Roeschlau P, Bernt E, Gruber W. Enzymatic determination of total cholesterol in serum. *Z Klin Chem Klin Biochem* 1974;12:226–230.
10. National Institutes of Health. Recommendations for improving cholesterol measurement: A report from the Laboratory Standardization Panel of the National Cholesterol Education Program. Bethesda, MD: National Institutes of Health; 1990. NIH publication no.: 90–2964.
11. McDade TW, Williams S, Snodgrass JJ. What a drop can do: Dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography* 2007;44:899–925.
12. Koal T, Burhenne H, Römling R, Svoboda M, Resch K, Kaever V. Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2005;19:2995–3001.

13. Mei JV, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr* 2001;131:1631S–1636S.
14. Lakshmy R, Reddy KS, Jaikhanani BL. Measurement of cholesterol and triglycerides in dried serum and the effect of storage. *Clin Chem* 2001;47:1113–1115.
15. Quraishi R, Lakshmy R, Prabhakaran D, Mukhopadhyay AK, Jaikhanani B. Use of filter paper stored dried blood for measurement of triglyceride. *Lipids Health Dis* 2006;5:20–22.
16. Quraishi R, Lakshmy R, Prabhakaran D, Irshad M, Mukhopadhyay AK, Jaikhanani BL. Effect of storage temperature on cholesterol measurement from dried blood. *Indian J Med Res* 2007;126:228–229.
17. Lakshmy R, Gupta R, Prabhakaran D, Snehi U, Reddy KS. Utility of dried blood spots for measurement of cholesterol and triglycerides in a surveillance study. *Diabetes Sci Technol* 2010;4:258–262.
18. DeBarber AE, Luo J, Star-Weinstock M, et al. A blood test for cerebrotendinous xanthomatosis with potential for disease detection in newborns. *J Lipid Res* 2014;55:146–154.
19. Methel AH, Hogg RC, Buzikievich LM, Stark KD. Butylated hydroxytoluene can protect polyunsaturated fatty acids in dried blood spots from degradation for up to 8 weeks at room temperature. *Lipids Health Dis* 2013;12:22–31.
20. Gelzo M, Dello Russo A, Corso G. Stability study of dehydrocholesterols in dried spot of blood from patients with Smith–Lemli–Opitz syndrome, using filter-paper treated with butylated hydroxytoluene. *Clin Chim Acta* 2012;413:525–526.
21. Gelzo M, Clericuzio S, Barone R, et al. A routine method for cholesterol and 7-dehydrocholesterol analysis in dried blood spot by GC–FID to diagnose the Smith–Lemli–Opitz syndrome. *J Chromatogr B Analyt Technol Biomed Life Sci* 2012;907:154–158.
22. Lacher DA, Berman LE, Chen T-C, Porter K. Comparison of dried blood spot to venous methods for hemoglobin A1c, glucose, total cholesterol, high-density lipoprotein cholesterol, and C-reactive protein. *Clin Chim Acta* 2013;422:54–58.
23. Lakshmy R, Mathur P, Gupta R, Shah B, Anand K, Mohan V, et al. Measurement of cholesterol and triglycerides from a dried blood spot in an Indian Council of Medical Research-World Health Organization multicentric survey on risk factors for noncommunicable diseases in India. *J Clin Lipidol* 2012;6:33–41.
24. Graham I, Atar D, Borch-Johnsen K, et al. European guidelines on cardiovascular disease prevention in clinical practice: Executive summary. *Atherosclerosis* 2007;194:1–45.
25. Chace DH, Millington DS, Terada N, Kahler SG, Roe CR, Hofman LF. Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. *Clin Chem* 1993;39:66–71.
26. Blanchard S, Sadilek M, Scott CR, Turecek F, Gelb MH. Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: Application to screening newborns for mucopolysaccharidosis I. *Clin Chem* 2008;54:2067–2070.
27. Fingerhut R, Ensenaer R, Röschinger W, Arnecke R, Olgemöller B, Roscher AA. Stability of acylcarnitines and free carnitine in dried blood samples: Implications of retrospective diagnosis of inborn errors of metabolism and neonatal screening for carnitine transporter deficiency. *Anal Chem* 2009;81:3571–3575.
28. la Marca G, Malvagia S, Materazzi S, et al. LC-MS/MS method for simultaneous determination on a dried blood spot of multiple analytes relevant for treatment monitoring in patients with tyrosinemia type I. *Anal Chem* 2012;84:1184–1188.
29. Coombes EJ, Gamlen TR, Batstone GF, Leigh PN. A phenytoin assay using dried blood spot samples suitable for domiciliary therapeutic drug monitoring. *Ann Clin Biochem* 1984;21:519–522.
30. Allanson AL, Cotton MM, Tetley JN, Boyter AC. Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: A potential method for therapeutic drug monitoring. *J Pharm Biomed Anal* 2007;44:963–969.
31. la Marca G, Malvagia S, Filippi L, et al. Rapid assay of topiramate in dried blood spots by a new liquid chromatography-tandem mass spectrometric method. *J Pharm Biomed Anal* 2008;48:1392–1396.
32. Tziakas DN, Kaski JC, Chalikias GK, et al. Total cholesterol content of erythrocyte membranes is increased in patients with acute coronary syndrome: A new marker of clinical instability? *J Am Coll Cardiol* 2007;49:2081–2089.
33. Gottlieb MH. Rates of cholesterol exchange between human erythrocytes and plasma lipoproteins. *Biochim Biophys Acta* 1980;600:530–541.