Comparison of three homogeneous methods for measuring high-density lipoprotein cholesterol with a precipitation method in diabetic and non-diabetic subjects

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ABSTRACT

Three homogenous methods for the automated measurement of high-density lipoprotein–cholesterol (HDL–C) are studied and compared with a precipitation method in diabetic and non-diabetic subjects. All three methods meet the precision criteria of the National Cholesterol Education Programme (NCEP). Triglycerides did not cause significant interference up to 10 mmol/L in the immuno-inhibition method (Wako) and up to 40 mmol/L in the Randox and Roche methods. Haemoglobin, up to a concentration of 5 g/L, had only negligible effect on the performance of all three homogeneous methods. Bilirubin caused an increasing positive bias in all methods above a concentration of 50 µmol/L. In comparison with the precipitation method, the new homogeneous methods agreed for type 1 diabetic patients but showed a positive bias for the control subjects and patients with type 2 diabetes. The bias of HDL–C levels in type 2 diabetes may be sufficient to affect the calculation of cardiovascular risk, and may therefore influence the decision to prescribe lipid-lowering medication.

KEY WORDS: Cardiovascular risk. Diabetes mellitus. HDL–cholesterol.

Introduction

Monitoring high-density lipoprotein–cholesterol (HDL–C) is clinically important because its level in plasma inversely correlates with the risk of cardiovascular disease.[1–3] Until recently, the common method for determining HDL–C was an indirect two-step procedure. This method involves addition of divalent cations and polyanions for the precipitation of apolipoprotein-B-containing particles, with subsequent cholesterol determination in the supernatant. However, this method is laborious, uses expensive staff time and is not amenable to automation.

Several methods have now been introduced for the direct measurement of HDL–C that are readily adaptable to automation.[4–8] These will make it easy to measure large numbers of HDL–C levels cheaply. However, it has been reported that some of the homogeneous methods overestimate HDL–C values.[9] Conversely, in liver cirrhosis, a condition associated with significant alteration in lipoprotein structure and composition, the homogeneous methods underestimate HDL–C concentration.[7,8]

Diabetes mellitus is a syndrome associated with significant disturbances of lipoprotein metabolism. HDL-cholesterol is generally low in type 2 diabetes mellitus and in poorly controlled type 1 diabetes. In patients who are well-controlled on insulin therapy, HDL–C concentration may be higher than in non-diabetic populations.[10,11]

In a preliminary study[12], we found that two of the homogeneous methods tend to overestimate HDL–C level compared to the precipitation method in a mixed group of diabetic samples. We have also shown that the bias in HDL levels in type 2 diabetes, as measured by the Roche assay, is likely to affect cardiovascular risk estimation and the decision to prescribe lipid-lowering medication.[13]

In this study we compare the performance of three homogeneous assays with a conventional precipitation method in samples from groups of type 1 and type 2 diabetic patients and from a group of control subjects. We also investigate the interference by triglycerides, bilirubin and haemoglobin in the homogeneous assays.

The precipitation method used in this study has been evaluated recently and compared with the Centers for Disease Control (CDC) reference method.[14] It was found to satisfy the criteria of the National Cholesterol Education Program for precision and accuracy.

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Materials and methods

Precipitation method

In the precipitation method, 200 µL serum was mixed with 500 µL precipitation reagent (phosphotungstic acid 0.55 mmol/L, $MgCl_2$ 25 mmol/L; Boehringer Mannheim). After 15-min incubation, samples were centrifuged (4000 x*g* for 15 min) and the supernatant was collected and HDL–C measured. Cholesterol was measured in the supernatant using the Roche Modular analyser and Roche Cholesterol reagent.

Table 1. Within-batch precision of three HDL–C methods, using QC material. Thirty aliquots of each QC sample were assayed by all methods.

		Roche	Randox	Wako
Low QC	Mean	0.800	0.726	0.790
	SD	0.011	0.007	0.009
	CV %	1.350	0.992	1.165
High QC	Mean	1.964	1.865	2.077
	SD	0.045	0.024	0.034
	CV %	2.312	1.265	1.637

Table 2. Between-batch precision with QC material. Thirty aliquots were assayed in different runs.[15]

		Roche	Randox	Wako
Low QC	Mean	0.752	0.670	0.729
	SD	0.021	0.018	0.014
	CV %	2.780	2.657	1.933
High QC	Mean	1.794	1.759	1.895
	SD	0.033	0.039	0.034
	CV %	1.817	2.200	1.768

Homogeneous methods

Three homogeneous methods were used in this study. Homogeneous methods rely on masking or inactivating the very low density lipoprotein (VLDL) and low-density lipoprotein (LDL), leaving only the HDL–C available for reaction with cholesterol esterase.

In the Wako method (Alpha Laboratories, Eastleigh, Hampshire, UK), anti-human β-lipoprotein antibody in reagent 1 (R1) binds to LDL, VLDL and chylomicrons, but not to HDL. The antigen–antibody complexes formed block enzyme reactions. Cholesterol esterase and cholesterol oxidase in reagent 2 (R2) react only with HDL–C.

In the first step of the Roche method (Roche Diagnostics, Bell Lane, Lewes, East, Sussex, UK), sulphated cyclodextrin and dextran sulphate form water-soluble complexes with LDL, VLDL and chylomicrons. This makes them resistant to PEG-modified enzymes. In the second reaction step, the HDL–C is determined using PEG-modified cholesterol esterase and PEG-modified cholesterol oxidase.

In the Randox method (Randox Laboratories, Crumlin, Co. Antrim, UK), special ionic strength buffer releases cholesterol from the non-HDL–C lipoprotein components and eliminates their cholesterol. In the second step, HDL–C is released by detergents and measured enzymatically.

The three homogeneous HDL–C assays were all run on the Roche Modular system.

Other measurements

The cholesterol, triglyceride and bilirubin methods were run following Roche specifications.

Precision checks for homogeneous methods

Within-batch and between-batch precision were assessed using lipid controls materials provided by Randox Laboratories (n=30). The quality control (QC) material was a lyophilised human serum-based reagent.

Interference studies

Interference from triglycerides, haemoglobin and bilirubin was assessed. A serum pool sample was supplemented with Intralipid, haemoglobin or bilirubin, and HDL–C was measured in triplicate. The serum pool had baseline values of 1.7 mmol/L for triglycerides and 8 μmol/L for bilirubin, and was free of haemolysis as indicted by a low haemolysis index.

Blood samples

Blood samples were obtained from 30 patients with type 1 diabetes, 30 patients with type 2 diabetes and 30 non-diabetic subjects. The diabetic patients all attended the diabetic clinic at Queen Elizabeth Hospital, Gateshead.

All samples used in the study were collected into Becton Dickinson SST Plus Gel tubes (Cat No. 367973). Samples were stored at 4°C and used within three days. Triglyceride level in all samples was <4.5 mmol/L.

Calculation of cardiovascular risk

The 10-year risk of coronary heart disease (CHD) was calculated using a computer program adopted by the National Service Framework (NSF) for prevention of CHD.[18] The NSF recommends prescription of lipid-lowering drugs for patients with a 10-year risk for CHD of >30% as the initial stage. The next stage is identification and treatment of subjects with a 10-year risk of >15%.

Table 3. Calculation of 10-year CHD risk when HDL–cholesterol was measured by either a homogeneous or a precipitation method.

	HDL-C concentration		10-year CHD risk (%)	
Total cholesterol	Homogeneous method	Precipitation method	Homogeneous method	Precipitation method
6	1.4	1.2	10.2	15
5.4	1.0	0.8	15.8	20.4
5.2	0.8	0.6	22.9	30

The risk is calculated for a hypothetical 56-year-old man, a non-smoker with type 2 diabetes and blood pressure 140/80 mmHg, using a computer program based on a Framingham equation. The computer program was adopted by the Joint British Guidelines for CHD prevention.[18] Comparison was done around cut-off levels of 15%, 20% and 30%. HDL–C concentrations were obtained using the regression equation relating results obtained by the precipitation method and a homogeneous method (Roche).

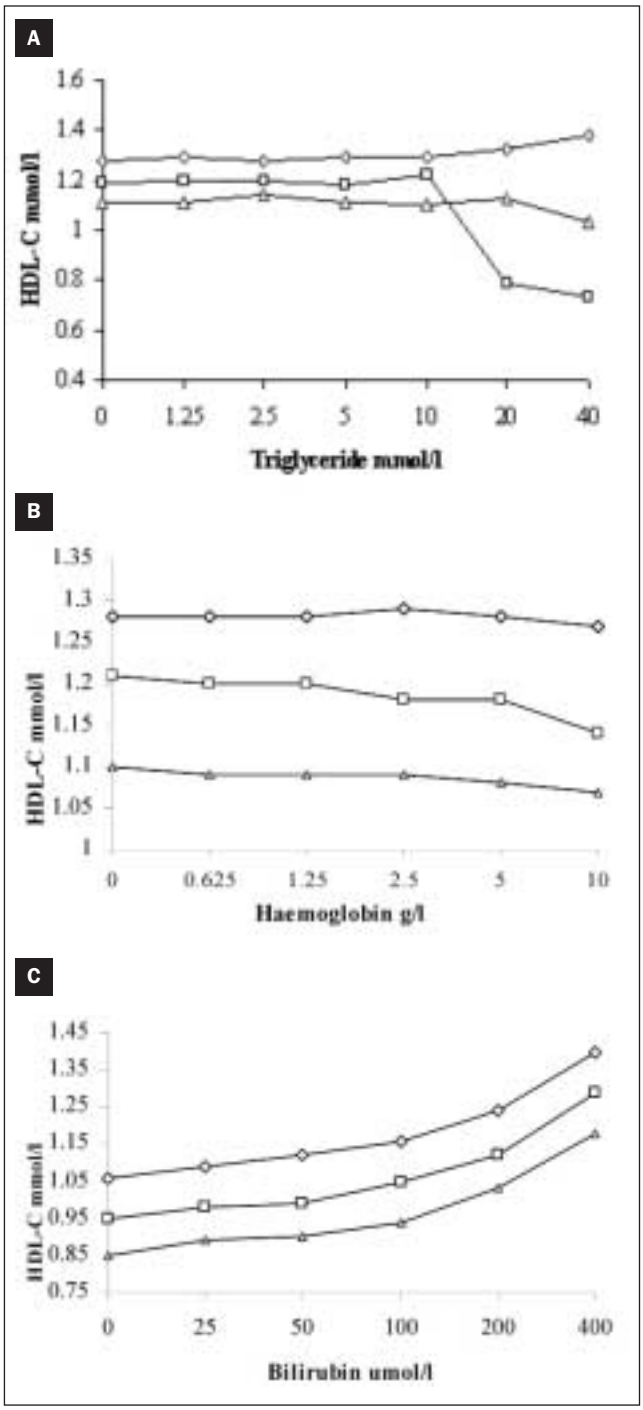

Fig. 1. Interference by triglycerides (A), haemoglobin (B) and bilirubin (C) with three homogeneous assays for HDL–C. (◊) Roche, (□) Wako, (Δ) Randox.

Statistical Analysis

Mean, standard deviation (SD) and coefficient of variation (CV) were calculated. Passing and Bablock regression was used for comparison studies.

Results

Precision values for homogeneous methods

The within-batch precision values for the three homogeneous HDL–C assays, using QC material, ranged from 0.99% to 2.31% (Table 1).

Table 2 shows the between-batch precision values for the three homogeneous HDL–C assays. All three methods satisfied the NCEP precision criteria guidelines of CV ≤ 4% at HDL-C concentration >1.09 mmol/L and SD <0.044 mmol/L for HDL–C <1.09 mmol/L.[14]

Interference in homogeneous methods

Triglycerides interference: All three methods showed little interference with triglycerides concentrations up to 10 mmol/L. The Wako method suffered a negative bias at higher triglyceride levels, while the Randox and Roche methods were more resistant. At triglycerides concentrations of 40 mmol/L, the Randox method suffered a negative bias of 7.2%, while the Roche method showed a positive bias of 7.8%.

Haemoglobin interference: All three assays showed good resistance to haemoglobin (Hb) interference and the negative bias was less than 3% at Hb concentration of 5 g/L. At an Hb concentration of 10 g/L, the Wako method showed a negative bias of 4.2%.

Bilirubin interference: All three homogeneous methods showed positive interference due to the presence of bilirubin, reaching approximately 10% at bilirubin concentration of 100 mmol/L and reaching 32–39% at bilirubin concentrations of 400 mmol/L.

Comparison with precipitation

All three homogeneous methods showed a positive bias when compared with the precipitation method, which was minimal for type 1 diabetes but worse for type 2 diabetes (Fig 2a, b and c). Average levels of bias, in the HDL–C concentration range of 0.5–2 mmol/L, for control subjects, patients with type 1 and patients with type 2 diabetes, respectively, were as follows: +0.18, +0.12 and + 0.21 for the Roche method; +0.12, 0.03 and 0.16 for the Randox method; and +0.19, 0.08 and 0.19 for the Wako method.

Discussion

The principles of each homogeneous assay studied are different and there are slight variations in the methods of measuring cholesterol. However, the performance of these methods showed many similarities, which permits some common conclusions to be drawn.

In agreement with most, but not all, reports, the homogeneous methods proved to be precise according to the NCEP criteria. All three methods were resistant to the effect of haemolysis and to triglycerides up to a concentration of 10 mmol/L. However, the positive interference by bilirubin makes these methods suitable only for bilirubin concentrations below 50 mmol/L.

In the groups studied here, the positive bias observed with the homogeneous methods was minimal in type 1 diabetic patients but worse in the type 2 diabetic patients. This pattern probably reflects the nature of the concentration dependence of the systematic bias, which is worse at lower concentrations of HDL–C but tends to correct itself at higher HDL–C concentrations. Treated type 1 diabetic patients tend

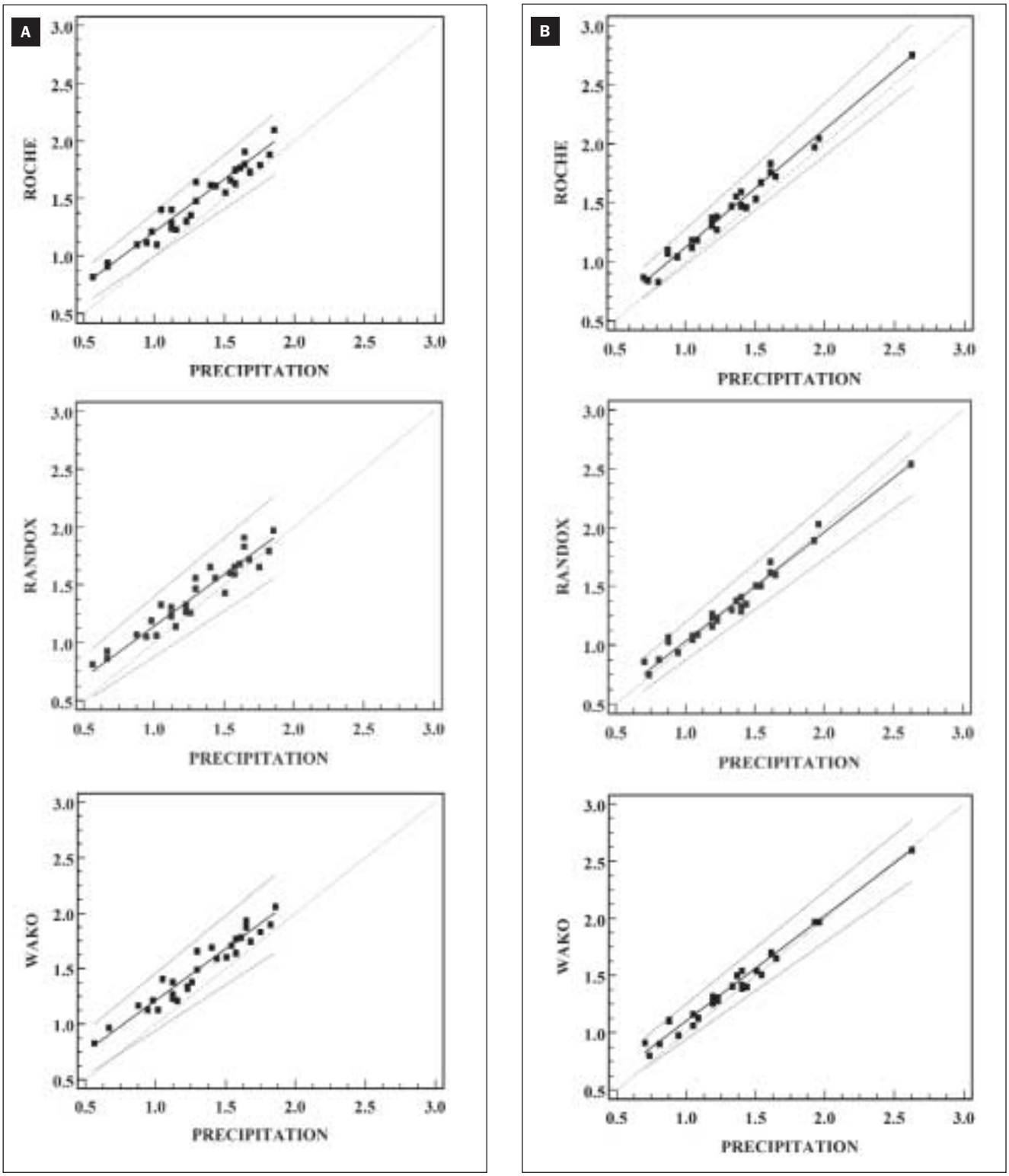

Fig. 2. Correlation of HDL–C concentration measured by the precipitation method and three homogeneous methods in non-diabetic subjects (A), patients with type 1 diabetes (B) and patients with type 2 diabetes (C, see next page). according to Passing and Bablock. The deviation between methods is shown by the deviation of the solid line (representing regression) from the line of equivalence (dashed). Also shown are the 95% confidence limits and the regression equations.

to have elevated HDL levels, while type 2 diabetic patients commonly have low HDL-C concentrations.

Many methods for cardiovascular risk assessment utilise a ratio of total cholesterol/HDL–C.[16–18] Overestimation of HDL-C by the homogeneous methods results in a lower cholesterol/HDL–C ratio and underestimation of cardiovascular risk. This effect is likely to be significant in type 2 diabetes, which is characterised by low HDL–C, and it could influence decisions about drug therapy in some patients (Table 3). Inaccuracy in HDL-C concentrations may

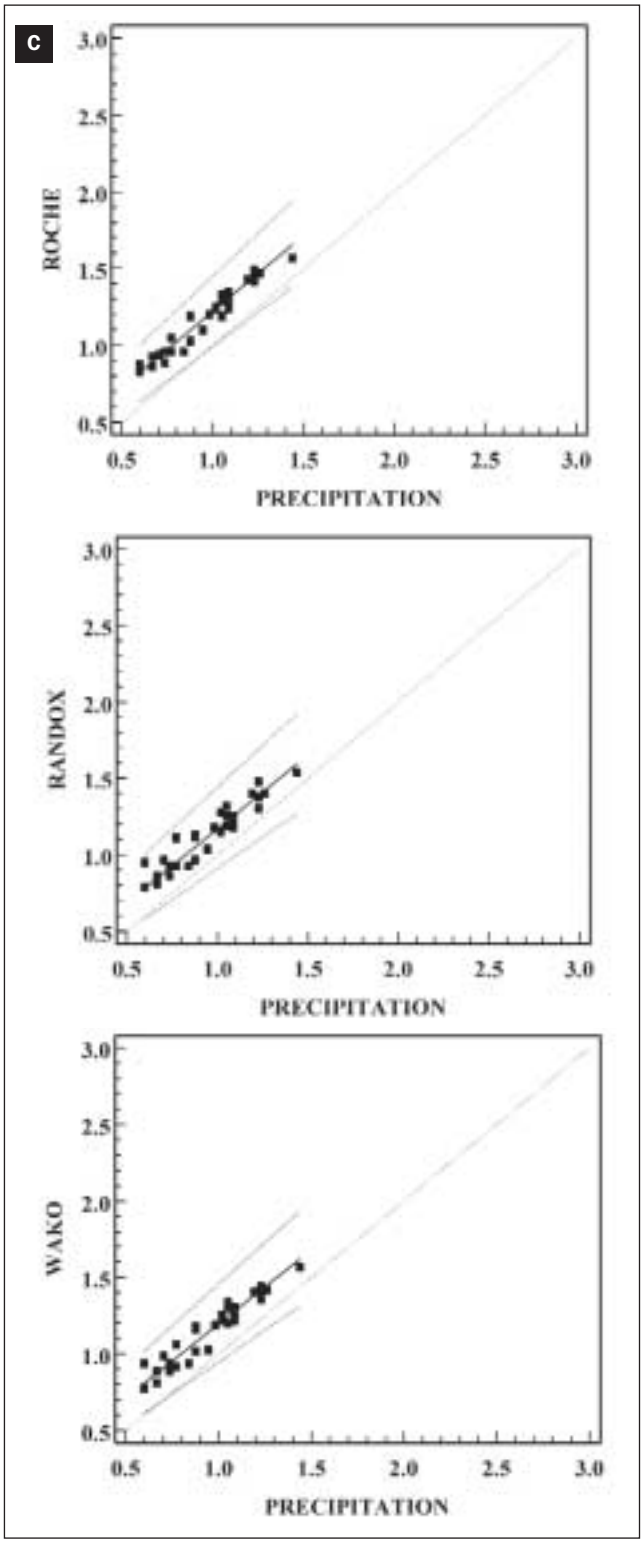

Fig. 2 (continued from previous page). Correlation of HDL-C concentration measured by the precipitation method and three homogeneous methods in patients with type 2 diabetes (C).

also cause erroneous estimation of LDL-cholesterol concentrations and may cause misclassification of patients according to level of cardiovascular risk.

It has already been shown that current Framingham equations underestimate cardiovascular risk in patients with type 2 diabetes.[19] Thus, the positive bias of direct HDL–C methods is an additional source of error in these calculations.

In conclusion, it has been shown that homogeneous methods for HDL–C measurement have acceptable precision. They are not influenced by triglyceride concentrations up to 10 mmol/L or Hb concentration up to 5 g/L. However, they are not suitable for use when bilirubin concentration is >50 μmol/L. These methods have questionable accuracy in both non-diabetic and diabetic samples, which may influence cardiovascular risk estimation and the decision to initiate lipid-lowering drug therapy, especially in type 2 diabetes. □

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