Clinica Chimica Acta 427 (2014) 86–93

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

# Development of a homogeneous assay for measurement of high-density lipoprotein-subclass cholesterol

Yasuki Ito [a,*], Noriyuki Satoh [a], Takayoshi Ishii [a], Junko Kumakura [a], Tsutomu Hirano [b]

[a] Reagent R&D department, Denka Seiken Co., Ltd., 1359-1, Kagamida, Kigoshi, Gosen-shi, Niigata 959-1695, Japan
[b] Department of Medicine, Division of Diabetes, Metabolism, and Endocrinology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan

ARTICLE INFO

*Article history:*
Received 22 May 2013
Received in revised form 11 September 2013
Accepted 11 September 2013
Available online 19 September 2013

*Keywords:*
HDL subclasses
HDL3: HDL3-C
Surfactant

ABSTRACT

*Background:* Several studies have suggested that measurement of high-density lipoprotein (HDL) 2 and HDL3 subfractions might be more useful for evaluating coronary risk than total HDL-cholesterol (C). However, methods of measuring HDL2 and HDL3 are quite laborious for general clinical use. Development of a quick and easy method of measuring HDL subfractions has been long-awaited.
*Methods:* Triglyceride (TG) rich lipoproteins (TRLs), low-density lipoprotein (LDL), HDL2, and HDL3 were used for screening of surfactants and enzymes to react selectively with HDL3-C and to decompose other lipoproteins.
*Results:* In order to develop HDL3-C homogeneous assay, polyoxyethylene styrenated phenyl ether derivative, for which the hydrophilic lipophilic balance (HLB) value is 13.6, was adopted as the most effective and specific surfactant for selection of HDL3 from HDL. Sphingomyelinase (SMase) reacted with TRLs and LDL preferentially, and decomposed them. HDL2-C was estimated by subtracting measured HDL3-C from total HDL-C, directly measured by homogeneous method. The homogeneous assay exhibited excellent correlations with the results of HDL3-C and HDL2-C measured by standard ultracentrifugation ($R^2 = 0.848$ and 0.982, respectively).
*Conclusions:* We established a rapid and effective, fully-automated assay for the measurement of HDL3-C. Furthermore, the subtraction of HDL3-C from total HDL-C allows concurrent determination of HDL2-C.


## 1. Introduction

High-density lipoprotein (HDL) has well characterized cardioprotective features such as its antioxidant and anti-inflammatory properties and the ability to efflux cholesterol from macrophages. HDL-cholesterol (C) is a negative risk factor for coronary heart disease (CHD). Low HDL-C increases the risk of CHD as powerfully as elevated low-density lipoprotein (LDL)-C. HDL consists of two major subclasses: large buoyant HDL2 (d = 1.063–1.125 kg/l) and small dense HDL3 (d = 1.125–1.210 kg/l). Several lines of evidence suggest that measuring HDL subclasses reflects CHD risk better than measurement of total HDL does; however, this is as yet inconclusive [1–6]. Detailed characterization of the subclasses, HDL2 and HDL3, is ongoing.

HDL2 and HDL3 were originally measured by ultracentrifugation [7] or gradient gel electrophoresis [8,9]. These methods are unsuitable for routine analysis of a large number of samples since they require complicated, time-consuming techniques. While recent methods such as capillary electrophoresis [10], HPLC [11], and nuclear magnetic resonance (NMR) [12] have diagnostic utility, each is limited because of the need for expensive equipment. Several study groups have developed an alternative method to measure HDL2-C and HDL3-C using precipitation [13–17]. This method requires the precipitation of VLDL and LDL fractions with heparin–manganese (Hep–Mn). The supernatant from this reaction is treated with dextran sulfate (DS) to precipitate HDL2. We previously developed a further simplified single-step Hep–Mn–DS precipitation method for HDL3-C, which concurrently removes HDL2 and apolipoprotein B-containing lipoproteins [18]. Even with this simplified method, skillful technique is required for the offline sample pretreatment. Thus, there remains a strong need for an easy and rapid method of measuring HDL2 and HDL3.

By screening surfactants and additives to react solely with HDL3, and by preventing triglyceride (TG) interference, we have succeeded in establishing a fully automated homogeneous assay for HDL3-C. Moreover, this same method allows quantification of HDL2-C levels by the subtraction of HDL3-C from total HDL-C. Herein we report the processes of the screenings, their purposes, and performance data of this novel assay.

## 2. Materials and methods

### 2.1. Materials

Cholesterol esterase from Asahi Kasei Pharma, cholesterol oxidase from Kikkoman Biochemifa, peroxidase from Toyobo, and catalase

*Abbreviations:* TRLs, TG rich lipoproteins; HLB, hydrophilic lipophilic balance; SMase, sphingomyelinase; Hep–Mn, heparin–manganese; DS, dextran sulfate; TOOS, *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dehydrate; BES, *N*,*N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; 4-AA, 4-Aminoantipyrine.

* Corresponding author. Tel.: +81 250 42 7109.
*E-mail address:* y_ito@denka-seiken.co.jp (Y. Ito).

0009-8981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2013.09.009

from Roche Diagnostics were used. *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate (TOOS) and *N*,*N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (Good's buffer (BES)) were from Dojindo Laboratories, 4-Aminoantipyrine (4-AA) from Nittobo, and sodium azide from Nacalai Tesque. We obtained sphingomyelinase (SMase) derived from *Streptomyces* sp. from Asahi Kasei Pharma and surfactants from following manufactures: Nippon Nyukazai, Kao, Aoki Oil Industrial, Dai-ichi Kogyo Seiyaku, Adeka, NOF, and Nacalai Tesque.

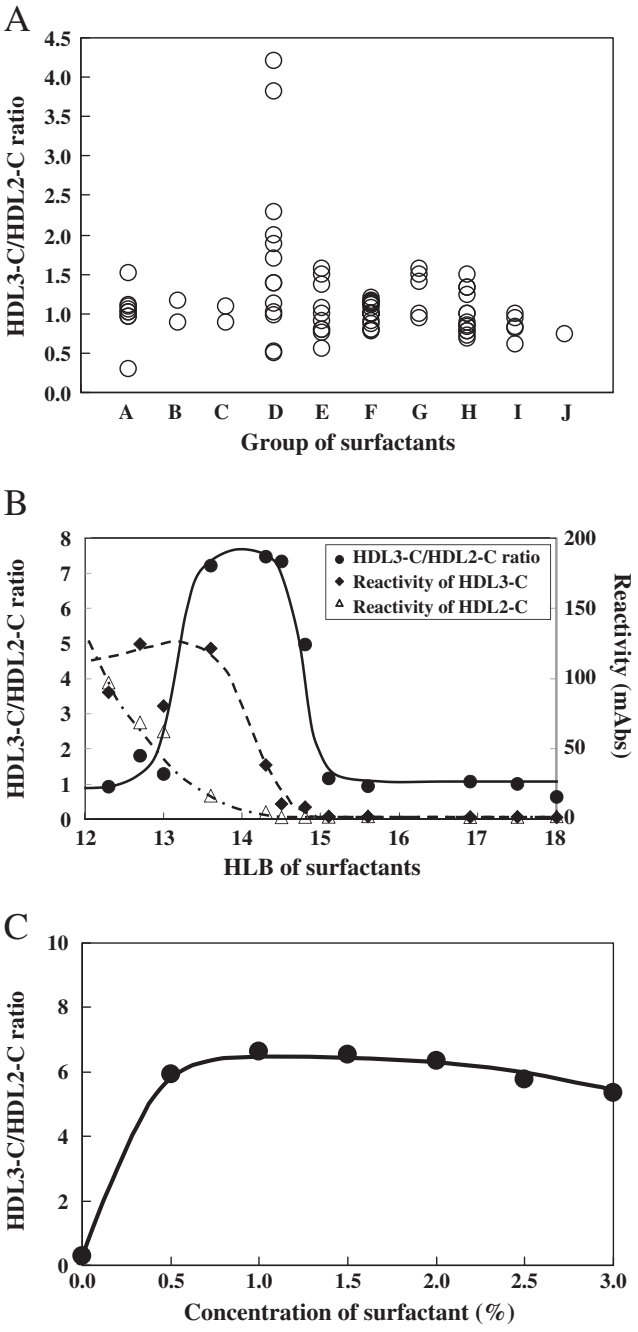


**Fig. 1.** Screening for HDL3 specific surfactant. A: Primary screening for HDL3 specific surfactants with polyoxyethylene alkyl ether (A), polyoxyethylene alkylene alkyl ether (B), polyoxyethylene alkyl phenyl ether (C), polyoxyethylene polycyclic phenyl ether (D), polyoxyethylene alkyl amine (E), polyoxyethylene polyoxypropylene block polymer (F), other non-ionic surfactants (G), anionic surfactants (H), amphoteric surfactants (I), and cationic surfactant (J). HDL3-C/HDL2-C ratio was calculated as the quotient of reactivity of HDL3-C (mAbs) by reactivity of HDL2-C (mAbs). High HDL3-C/HDL2-C ratio indicates that the HDL3 reacts superior to the HDL2 (n = 2). B: Reactivity of polyoxyethylene polycyclic phenyl ether derivatives with the range of hydrophilic lipophilic balances (HLB) 12.0–18.0 to reactivity of HDL2-C (mAbs), reactivity of HDL3-C (mAbs), or the HDL3-C/HDL2-C ratio (n = 2). C: Dose–response curve of the polyoxyethylene styrenated phenyl ether derivative with HLB 13.6 (surfactant) on the HDL3-C/HDL2-C ratio (n = 3).

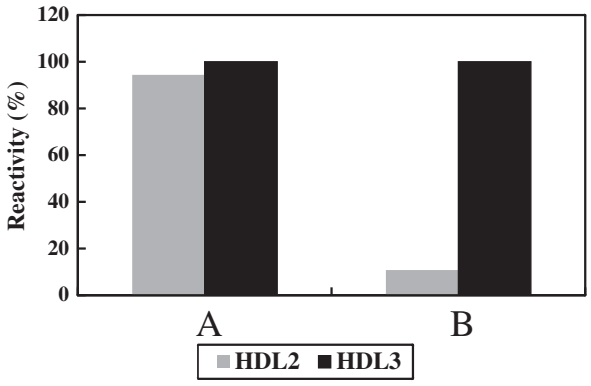


**Fig. 2.** The reactivity of the polyoxyethylene styrenated phenyl ether derivative to HDL2 and HDL3. The optimized polyoxyethylene styrenated phenyl ether derivative (B) selectively reacts with HDL3, whereas the polyoxyethylene benzylphenyl ether derivative (A) used as a total HDL-C assay (HDL-EX) reacts equally with both HDL2 and HDL3. Reactivity (%) was calculated as the quotient of reactivity of each fraction (mAbs) by reactivity of HDL3-C (mAbs) (n = 5).

### 2.2. Measurements

Total-C and HDL-C were measured with commercially available test kits (T-CHO and HDL-EX, respectively; Denka Seiken). HDL3-C was measured using our previous method: combined single precipitation using Hep–Mn–DS and homogeneous HDL-C assay [18]. HDL2-C levels were determined by subtracting HDL3-C from HDL-C.

### 2.3. Separation of lipoproteins: TRL, LDL, HDL2 and HDL3 by ultracentrifugation

TG-rich lipoproteins (TRLs) (density < 1.019 kg/l), LDL (d = 1.019–1.063 kg/l), HDL2 (d = 1.063–1.125 kg/l), and HDL3 (d = 1.125–1.210 kg/l) were separated from serum by sequential flotation in an ultracentrifuge (Himac CS120GX, Hitachi Koki) and S100AT6 rotor (Hitachi Koki) according to the method of Havel et al. [19]. Lipoprotein-C from ultracentrifugation fractions was measured using

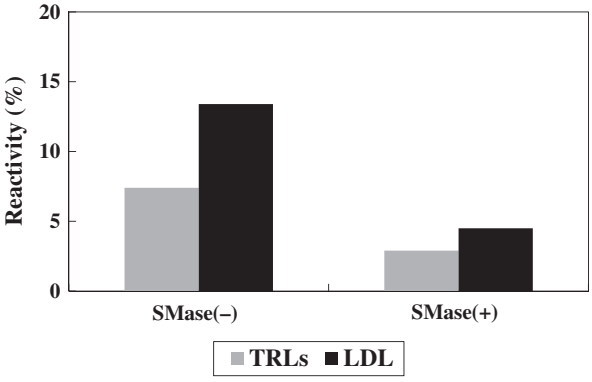


**Fig. 3.** The effect of sphingomyelinase (SMase) on the reactivity to TRLs and LDL with the polyoxyethylene styrenelphenyl ether derivative. Additional SMase minimizes the cross-reaction between TRLs and LDL to <5%. Reactivity (%) was calculated as the quotient of reactivity of each fraction (mAbs) by reactivity of HDL3-C (mAbs) (n = 5).