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Use of Cyclodextrin–Cholesterol Complex as a Primary Standard in Cholesterol Analysis

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Cholesterol is an analyte commonly measured in various biological fluids. Enzymic procedures have virtually replaced the chemical methods for cholesterol determinations. Standardization of cholesterol assays often involve the use of solutions of this steroid containing organic solvents and/or detergents, or alternatively, the use of secondary standards. The present report shows the preparation of a primary cholesterol standard by its complexation with a chemically modified cyclodextrin, hydroxylpropyl β-cyclodextrin. The use of cyclodextrin for cholesterol solubilization is not deleterious to the enzymes and other constituents present in reagents for cholesterol analysis. Such a standard is biocompatible, stable, and safe and offers an excellent alternative to the utilization of organic solvents or detergents.

INTRODUCTION

Cholesterol analysis is commonly performed in biochemical research and in clinical laboratories. Due to their simplicity and precision, enzymic methods for cholesterol determination are now widely used (*1*). A number of problems plague the quantitation of this analyte in biological fluids such as serum, thus resulting in poor precision and accuracy. One such problem is the lack of cholesterol calibrators and quality control solutions that closely mimic cholesterol in the biological matrix. For example, in blood, cholesterol is found complexed by weak forces to other lipids and proteins forming lipoprotein particles which are suspended in an aqueous matrix. Although highly purified cholesterol is readily available, enzymatic methods are usually calibrated using secondary standards. Preparation of primary cholesterol standards has been problematic due to the low solubility of the steroid in water. Primary cholesterol standards are presently prepared by dissolving pure cholesterol in organic solvents such as isopropanol and *N,N*-dimethylformamide or in detergents such as hydroxypolyethoxydodecane (*2*). The use of organic solvents leads to safety problems due to their hazardous properties. In addition, organic solvents and detergents can also lead to inhibition of enzymes in the reaction mixture (*3*). For these reasons, secondary standards employing assayed biological material have become popular in the calibration of enzymic cholesterol assays. Although secondary standards have matrix properties similar to that of the specimen, there are some serious problems with their use. They must have an accurate target value which should be established by an accepted

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reference technique. In addition, such standards pose potential health hazards, should be stored refrigerated, and are expensive.

The present report demonstrates the preparation and evaluation of a primary aqueous cholesterol standard without using organic solvents or detergents. A chemically modified cyclodextrin (hydroxylpropyl β-cyclodextrin) is used for the solubilization of cholesterol. Since cyclodextrins (CDs) are naturally occurring macrocyclic glucose compounds, the proposed composition is biocompatible and safe.

MATERIALS AND METHODS

Chemicals and Reagents

Hydroxypropyl beta cyclodextrin was obtained from American Maize-Products Company (Hammond, IN). Cholesterol was obtained from Eastman Kodak Company (Rochester, NY). Cholesterol reference serum (MULTI-SET) was obtained from Curtin Matheson Scientific, Inc. (Houston, TX). Reagent for manual cholesterol determination was obtained from Sigma Chemical Company (St. Louis, MO).

Methods

1. Preparation of CD–cholesterol standard. The cholesterol standard was prepared by adding cholesterol powder to a 20% (w/v) solution of hydroxylpropyl β-cyclodextrin in distilled water (containing 0.5 g/liter sodium azide as preservative). The mixture was continually stirred at room temperature until a clear solution was obtained.

2. Cholesterol determinations. Kinetics of cholesterol assay reaction was monitored on a Shimadzu UV-160 Spectrophotometer using Sigma Cholesterol Procedure No. 352. In this procedure, cholesterol esters are first hydrolyzed by cholesterol esterase (EC 1.1.3.6) to cholesterol. The cholesterol produced by hydrolysis is oxidized by cholesterol oxidase (EC 3.1.1.13) to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide produced is then coupled with the chromogen, 4-aminoantipyrine and *p*-hydroxybenzenesulfonate in the presence of peroxidase (EC 1.11.1.7) to yield a quinoneimine dye whose absorbance is measured at 500 nm.

The CD–cholesterol standard was also analyzed on the Hitachi 704 Chemistry Analyzer using the CHOD-PAP (High Performance) cholesterol reagent (Boehringer-Mannheim Diagnostics, Indianapolis, IN). The principle of this assay reaction was similar to that of the Sigma reagent. The instrument was calibrated by a secondary standard (Precical, lyophilized pooled human sera) obtained from Boehringer-Mannheim.

RESULTS AND DISCUSSION

Many important biomolecules have very low solubilities in aqueous solutions. Numerous approaches have been taken to utilize these compounds as calibrators in biochemical and clinical analysis. Two common strategies are the use of water-soluble organic solvents and the addition of detergents.

More recently molecular encapsulation with cyclodextrins has been used for enhancing solubilities of hydrophobic compounds (*4*). CDs are cyclic, nonreducing glucose oligosaccharides. Commonly used cyclodextrins have six, seven, or eight glucopyranose units (*5*). They are often referred to as α-, β-, or γ-cyclodextrins, respectively. These doughnut-shaped structures have a hydrophobic cavity, the size of which increases with the number of glucose units. Host molecules form inclusion complexes with CDs by partial or complete inclusion into their cavity. Cavity size is thus an important criteria in cyclodextrin complexation. The internal diameter of beta-CD is about 7.8 Å, which is ideal for steroids (*6*). β-cyclodextrin has a very low water solubility (1.85 g/100 ml). The hydroxypropyl derivative, however, has a solubility greater than 60 g/100 ml. The preparation of a 2 g/liter cholesterol standard required a 20% (w/v) solution of hydroxypropyl beta-CD in water. Preparation of cholesterol calibrators with higher steroid concentrations will require greater amounts of cyclodextrin. The standard was prepared at room temperature. No heating was required. The resulting solution was stored at room temperature and remained optically clear throughout the duration of the study (about 1 month).

Kinetics displayed by the CD–cholesterol standard in a conventional cholesterol assay reagent (Sigma Cholesterol Procedure No. 352) was compared with a secondary standard (reference serum) and human serum. Results of this experiment, performed at room temperature, are shown in Fig. 1. Curve C (Fig. 1) shows the color formation for a reference serum (MULTI-SET). This is a serum-based liquid multianalyte calibrator which is used to calibrate chemistry analyzers. Reaction of human serum with the cholesterol reagent is shown as curve S (Fig. 1). Reaction of increasing amounts of CD–cholesterol standard with the cholesterol reagent are indicated by curves 1, 2, and 3, respectively (Fig. 1). In all cases, the reactions reached a stable endpoint before the manufacturer recommended incubation time of 18 min at ambient temperature. Comparison of the reaction kinetics and final absorbances obtained in curve C (reference serum; cholesterol concentration, 1.93 g/liter) with that of curve 1 (CD–cholesterol standard, cholesterol concentration, 2.20 g/liter) suggests that the hydroxypropyl beta-CD does not inhibit the enzymes present in the cholesterol reagent or affect the quinoneimine dye obtained in the reaction. The latter was an area of concern since cyclodextrins are known to alter the spectral characteristics of various dyes (*7*). Addition of increasing amounts of the CD–cholesterol standard resulted in a good linear response (cholesterol concentration of 2.20 g/liter for curve 1; cholesterol concentration of 4.40 g/liter for curve 2; and cholesterol concentration of 8.80 g/liter for curve 3; Fig. 1), thus demonstrating its potential use for instrument calibration.

Further evaluation of the CD–cholesterol solution was carried out on a routine chemistry analyzer. Accuracy and precision studies were performed on the Hitachi 704 using a reliable and accurate commercial cholesterol reagent (CHOD-PAP High Performance). This instrument was calibrated by a secondary standard that is traceable to the CDC reference method for cholesterol. A primary CD–cholesterol standard solution (cholesterol concentration, 1.95 g/liter) was analyzed 20 times for accuracy and within-run precision (Table 1). The mean of these

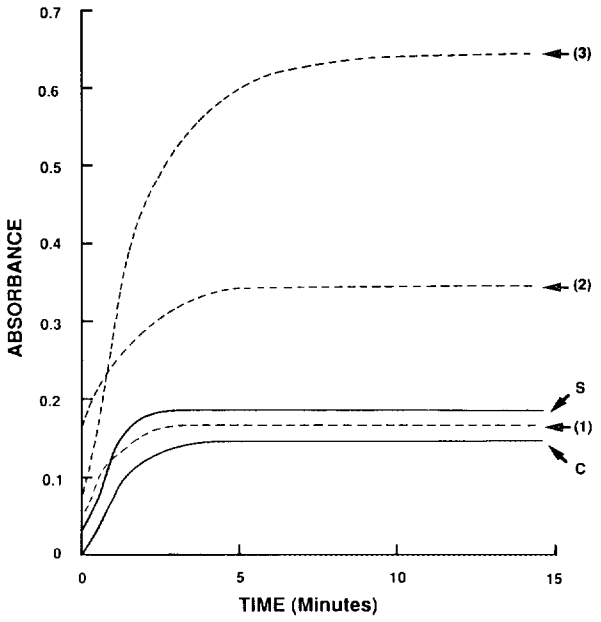

FIG. 1. Kinetics of the CD–cholesterol standard with a conventional cholesterol reagent are shown as curves 1, 2, and 3 (dotted lines). Reaction of human serum and reference serum are shown as curve S and curve C, respectively. Incubation temperature in all experiments was 24°C. Sample:reagent ratios were 1:101 for curves 1, S, and C. For curves 2, and 3, the sample:reagent ratios were 1:51 and 1:26, respectively.

measurements was 1.96 g/liter thus giving a bias of only 0.01. The within-run precision was excellent (coefficient of variation of less than 1%).

Between-run precision was performed by assaying the CD–cholesterol solution once a day for 20 days. The mean of these results was 1.96 g/liter. The bias was again very small (less than 1% of the actual value). The coefficient of variation was about 1%. The good day-to-day reproducibility of the primary standard indicates the stability of the CD–cholesterol material, which was stored at ambient temperature throughout the investigation. One of the beneficial properties of cy-

TABLE 1
Analysis of CD-Cholesterol Standard (1.95 g/liter) on the Hitachi 704 Chemistry Analyzer

n^a	Mean (g/liter)	S.D.[b] (g/liter)	C.V.[c] (%)	Minimum (g/liter)	Maximum (g/liter)
			Within-run		
20	1.96	0.01	0.76	1.93	1.99
			Between-run		
20	1.96	0.02	1.06	1.91	2.00

[a] Number of assays.
[b] Standard deviation.
[c] Coefficient of variation.

clodextrins, that has been used extensively in agriculture, cosmetics, and pharmaceuticals, is their ability to improve stability of various molecules (*8*). This advantage is also realized in its proposed use for the preparation of calibrators.

The studies above demonstrate the potential use of cyclodextrins in the preparation of primary standards for biochemical and clinical analysis. Calibrators of other hydrophobic bioanalytes can similarly be solubilized using an appropriate cyclodextrin. Molecular encapsulation offers an excellent alternative for the preparation of primary standards involving hydrophobic analytes.

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