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Automatic gas chromatographic determination of the high-density-lipoprotein cholesterol and total cholesterol in serum

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Abstract

A new analytical method that combines on-line precipitation–filtration, enzymatic hydrolysis, extraction and gas chromatography was developed for the determination of total cholesterol and high-density-lipoprotein cholesterol in human serum. Very-low-density lipoprotein, intermediate-density lipoprotein and low-density lipoprotein are precipitated with sodium phosphotungstate and magnesium chloride; then, the serum is continuously filtered and unprecipitated high-density-lipoprotein cholesterol is enzymatically hydrolyzed and finally determined as cholesterol by gas chromatography. Total cholesterol is also determined by direct introduction of the serum into the proposed system. The proposed method was validated by analyzing a lipid control serum with certified contents of high-density-lipoprotein cholesterol and total cholesterol. The results obtained were consistent with the certified contents.

1. Introduction

Heart disease is the number one cause of death in the world; epidemiological studies have shown a strong correlation between coronary heart disease and blood cholesterol levels. Lipids are carried by means of a series of rather complex micellar structures (lipoproteins). There are five major lipoprotein classes as regards density, namely: chylomicron, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). A number of underlying disease states of environmental influence are associated with reduced HDL levels, one of the most common associations being with hypertriglyceridemia [1,2].

Diagnostic tests for lipoprotein are usually performed in clinical laboratories. However, the determination of HDL cholesterol (HDL-C) is extremely difficult and impractical by direct means. Therefore, most available methods for this purpose measure the plasma content of HDL-C after selective precipitation of VLDL and LDL with various polyanions and divalent metals. These manual precipitation and centrifugation steps are only relatively accurate and precise; also, measurements of supernatant cholesterol are prone to significant oscillations because of the low HDL-C concentration usually involved [2]. Other methods for determining HDL-C involve a separation process such as ultracentrifugation [3], electrophoresis [4] (which

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is scarcely precise or accurate in the range of greatest clinical interest, viz. 20–40 mg/dl), and ion-exchange and gel-permeation chromatography (which are too complex and time-consuming for routine analyses) [2]. Therefore, despite the available alternatives, precipitation with polyanions and divalent metals for separation of the HDL-C fraction from other lipoproteins continues to be the preferred choice [5–11].

The determination of total cholesterol in serum is easier than that of HDL-C, hence the large number of methods available for the former analyte. Most of them are based on an enzymatic reaction and spectrophotometric and fluorimetric detection [12,13]. The instability and high cost of enzymes has fostered the development of immobilization methods; immobilized enzymes are packed into minicolumns that are then inserted into a continuous-flow system [14–17]. Cholesterol is often determined by gas chromatography (GC) [18–21] and high-performance liquid chromatography (HPLC) [22,23].

The aim of this work was to develop a new automatic method for the sequential determination of HDL-C and total cholesterol (the two lipid parameters of greatest interest in clinical chemistry) in serum. The HDL-C fraction was first separated from the other types of cholesterol by precipitation with a classical reagent of wide use in clinical laboratories, then hydrolyzed by using an immobilized cholesterol esterase enzyme reactor, and finally determined by GC. Total cholesterol in the serum sample was directly hydrolyzed and determined by GC.

2. Experimental

2.1. Materials

n-Hexane, petroleum ether, Triton X-100, 1,4-dioxane, ethyl acetate, ethyl ether, ethanol, 2-propanol and methanol were supplied by Merck (Darmstadt, Germany). All other reagents [sodium cholate, piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer, magnesium chloride, sodium phosphotungstate, cholesterol, cholesterol esterase, stigmasterol, Tris buffer, hydrochloric acid, sodium hydroxide, potassium phosphate, glutaraldehyde and 3-aminopropyltriethoxysilane] were obtained from Sigma (St. Louis, MO, USA). The controlled pore glass used as support for immobilizing the enzyme was purchased from Electronucleonics (Fairfield, NY, USA); 80/120 mesh, with an average pore size of 2025 Å. Serum samples were obtained from donors with a negative serological test. A lipid control serum sample for in vitro diagnostic use (diluted according to the laboratory's recommendations) for optimization of HDL-C isolation and construction of the calibration graphs for total cholesterol (Roche, Madrid, Spain), and a Roche control serum N (equine) for validation of the proposed method were also employed. The enzyme was stored at 4°C as per manufacturer's instructions.

A standard stock solution containing 1 g/l cholesterol was prepared in petroleum ether and stored in PTFE bottles at 4°C. The optimum GC conditions were determined by using a standard containing 50 mg/l cholesterol and 40 mg/l stigmasterol (internal standard) in petroleum ether. A 50 mmol/l PIPES buffer solution also containing 6 mmol/l sodium cholate at pH 8 was prepared. The extractant used was petroleum ether–ethanol (1:1) containing 40 mg/l stigmasterol as internal standard. Solutions of 40 g/l sodium phosphotungstate and 2 mol/l magnesium chloride (precipitating reagents) were used for the HDL-C determination.

2.2. Apparatus

Analyses were carried out on a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector and a 10 m × 0.53 mm I.D. fused-silica capillary column coated with a 2.65-μm film of 100% cross-linked poly(dimethylsiloxane) (HP-1). Nitrogen was used as the carrier gas at a flow-rate of 37.8 ml/min. The injector port and detector temperature were kept at 300°C. The oven temperature was raised from 235°C (2 min) to 250°C (5 min) at 3°/min. Peak areas were measured by means of a Hewlett-Packard 3392-A integrator.

Two Gilson-Minipuls-2 peristaltic pumps fitted with poly(vinyl chloride) and Solvaflex pumping tubes for aqueous and organic solutions, respectively, were used. An injection valve (Rheodyne 5041), a switching valve (Rheodyne 5301), PTFE tubing (0.5 mm I.D.) for coils, and a custom-made T-shaped glass separator housing with an internal PTFE tube were used. A 0.5–0105 column (Scientific Systems, Pennsylvania, USA) furnished with a removable screen-type stainless-steel filter (pore size 0.5 μm, chamber inner volume 580 μl, filtration area 3 cm^2) was used for filtration. A six-port switching valve (Knauer 6332000) mounted over the injection port of the gas chromatograph (injection volume 5 μl) was also employed [24].

2.3. Preparation of the immobilized enzyme reactor

Cholesterol esterase (100 U/ml, from *Pseudomonas fluorescens*) was immobilized on controlled pore glass by cross-linking with glutaraldehyde as previously reported by Massom and Townshend for glucose oxidase [25]. Enzyme reactors of different lengths and 1.0 mm I.D. were prepared from PTFE tubing and stored in a 0.1 mol/l potassium phosphate buffer of pH 7.0 at 4°C.

2.4. Procedure

The manifold used for the sequential determination of HDL-C and total cholesterol in serum is depicted in Fig. 1. In the HDL-C determination step, 0.5 ml of serum (adjusted to pH 7.5 with 0.1 mol/l HCl) plus precipitating reagents (150 μl of 40 g/l sodium phosphotungstate and 37.5 μl of 2 mol/l magnesium chloride) was propelled through the filter at 0.5 ml/min. The unprecipitated fraction of HDL-C was merged with a stream of 6 mmol/l sodium cholate in 50 mmol/l PIPES buffer (pH 8) at 0.5 ml/min. The mixture was continuously circulated through the injection valve (IV_1) and the loop contents (120 μl) were injected into the carrier (50 mmol/l PIPES, pH 8) at 0.5 ml/min. HDL-C was then hydrolyzed to cholesterol in the immobilized enzyme reactor (IMER) containing cholesterol esterase at 42°C. The stream emerging from the IMER was mixed with another carrying petroleum ether–ethanol (1:1) at 0.5 ml/min; then, lipoprotein micelles were disrupted and cholesterol was extracted in the 200-cm coil. The extract from the phase separator (T-type, glass-PTFE) was continuously circulated through the injection valve (IV_2), and the loop contents (the largest cholesterol fraction was located 5 min after IV_1 was switched) was injected into the nitrogen carrier stream and transferred to the

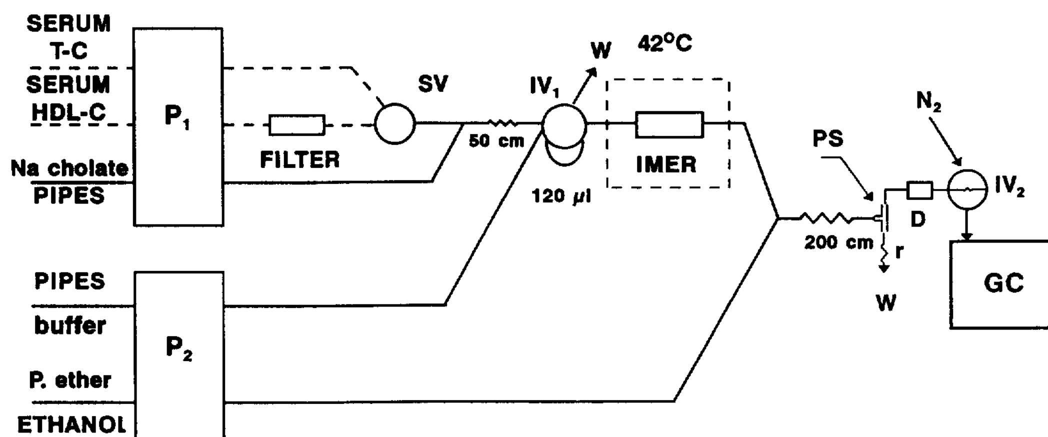

Fig. 1. Schematic diagram of the system used for the determination of total cholesterol (T-C) and high-density-lipoprotein cholesterol (HDL-C) in serum samples. P = pump; SV = switching valve; IV = injection valve; IMER = immobilized enzyme reactor; PS = phase separator; r = restrictor; D = desiccating column; W = waste; GC = gas chromatograph.

chromatograph port. The restrictor (r) used allowed virtually complete phase separation. A desiccating column (D, 50 mm × 3 mm I.D.) packed with sodium aluminosilicate pellets was used prior to IV_2 to prevent any water from reaching the column. Total cholesterol was determined by introducing 0.5 ml of diluted serum sample (0.1 ml of serum plus 0.4 ml of 0.15 mol/l NaCl) into the system via the switching valve (SV); the operating sequence was analogous to that described above for HDL-C determination.

3. Results and discussion

3.1. Isolation of HDL cholesterol

Classical methods for the determination of HDL-C use precipitation procedures [5–11]. For clinical purposes, the precipitating reagent must form an insoluble complex with all plasma lipoproteins except HDL, so that it remains in the supernatant and can be quantitated by its cholesterol content after centrifugation. Precipitating reagents containing sodium phosphotungstate-Mg(II) and dextran sulphate-Mg(II) have been reported to be more stable and compatible with enzymatic assays for cholesterol [5]. Other proposed reagents raise problems arising from small (but significant) amounts of HDL precipitating or from incomplete precipitation of other lipoproteins [2]. Therefore, we chose to use sodium phosphotungstate plus magnesium chloride as the precipitating reagent [8,9].

The official method that uses magnesium chloride and sodium phosphotungstate as the precipitating reagents adds these two in sequence [9]. In order to simplify the procedure, a mixture of the two reagents (40 g/l sodium phosphotungstate plus 2 mol/l magnesium chloride) was introduced into the flow system at a flow-rate of 0.2 ml/min and then merged with the serum control sample (the HDL-C and total cholesterol contents of which were 90 ± 9 mg/dl and 319 ± 17 mg/dl, respectively) at 0.5 ml/min. We tested various precipitation coil lengths in order to optimize precipitation since the manual method involves 30 min standing followed by centrifugation for a further 30 min. Cholesterol in the filtrate (collected from the filter at the end of the precipitation coil) was hydrolyzed manually with potassium hydroxide in methanol at 70°C for 60 min [20]; then, free cholesterol was extracted with *n*-hexane and 2-μl aliquots were injected into the chromatograph for analysis. The HDL-C fraction was quantified from calibration graphs constructed by using the same serum and manual hydrolysis and extraction of total cholesterol as for HDL-C. The joint addition of the two precipitating agents did not ensure complete precipitation of the corresponding cholesterol fraction, so the HDL-C contents obtained were overestimated. In order not to complicate the manifold used for sequential insertion of the precipitants (which would also involve diluting the serum sample), we chose to introduce them manually, thereby minimizing sample dilution. We then determined the optimum amount of precipitant. For this purpose, we tested adding variable volumes (40–280 μl) of 40 g/l sodium phosphotungstate to 0.5 ml of the control serum and, after shaking, variable volumes of 2 mol/l $MgCl_2$ (10–70 μl). A precipitant (sodium phosphotungstate/magnesium chloride) ratio of 4:1 was maintained throughout as per the recommendations for the manual procedure [9]. In order to ensure that the resulting volume (0.5 ml of the serum plus the volumes of the two precipitants, i.e. 0.85 ml) was preserved throughout the experiments, it was replenished with 0.15 mol/l NaCl as required. As can be seen in Fig. 2, the concentration of HDL-C (determined as cholesterol) diminished with increasing total volume of precipitant up to 140 μl because precipitation of low-lipoprotein cholesterol was favoured; above this volume, the concentration of cholesterol remained constant and was consistent with the certified HDL-C concentration in the serum control sample (90 mg/dl). A total volume of 187.5 μl (150 μl of 40 g/l sodium phosphotungstate plus 37.5 of 2 mol/l magnesium chloride) was selected for precipitating total cholesterol (HDL-C excluded).

The effect of the serum pH on the precipitation of total cholesterol minus HDL-C was studied over the range 5–8.5 (adjusted with

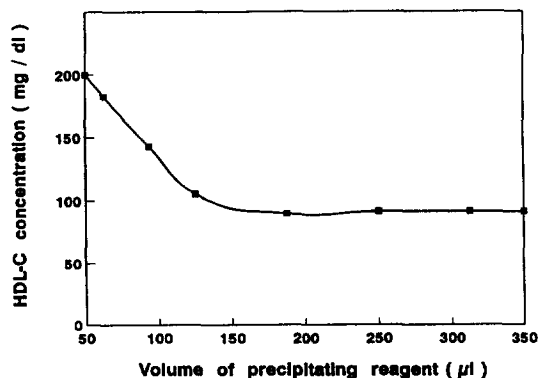

Fig. 2. Influence of the total volume of precipitating reagents (40 g/l sodium phosphotungstate and 2 mol/l magnesium chloride in a 4:1 volume ratio) on the precipitation of total cholesterol (HDL-C fraction excluded).

microvolumes of HCl or NaOH); the analytical signal remained virtually constant between 7.3 and 7.7, above which it decreased slightly, probably because some of the HDL-C fraction coprecipitated with the other types of cholesterol in the presence of precipitating reagent. Therefore, a pH of 7.5 was selected for further experiments. Two types of filters (PTFE, chamber inner volume 100 μl, filtration area ca. 3 cm^2; and stainless-steel, chamber inner volume 580 μl, and filtration area ca. 3 cm^2) were assayed for the continuous filtration of the manually precipitate formed by mixing 0.5 ml of serum with the precipitating reagents. The filtrate was hydrolyzed and the cholesterol extracted as described above [20]. Of the two filters tested, the stainless-steel filter proved to be more effective over a longer period as its chamber inner volume was higher than that of the PTFE filter, so the former was selected. The flow-rate of precipitated serum for filtration had no effect over the assayed range (0.1–0.8 ml/min) because filtration was instantaneous; a flow-rate of 0.5 ml/min was thus chosen.

3.2. Optimization of variables affecting the enzymatic hydrolysis of cholesterol esters

In these experiments, a pool serum sample diluted five times with 0.15 mol/l NaCl was employed. Cholesterol can be quantitated by both enzymatic and chemical methods [2]. We first assessed a chemical method for hydrolyzing cholesteryl esters; for this purpose, a stream of 1 mol/l potassium hydroxide in methanol was merged at a flow-rate of 0.3 ml/min with the diluted serum sample. Once hydrolysis was complete, free cholesterol was continuously extracted with n-hexane and the organic phase collected in 1-ml vials for subsequent injection of 2-μl aliquots into the chromatograph port. The hydrolysis reaction requires heating at 70°C for 60 min in the manual procedure; establishing these conditions in the flow manifold entailed stopping the flow for over 30 min, time during which proteins precipitated from the serum sample, thereby clogging connectors and the membrane of the phase separator as the system was restarted. We therefore abandoned the chemical method and tested an enzymatic method for hydrolyzing cholesterol.

In the enzymatic method (the most widely used in clinical laboratories), cholesteryl esters are quantitatively hydrolyzed to free cholesterol and fatty acids by cholesterol esterase [12–17]. Owing to the instability and high cost of cholesterol esterase, the enzyme was first immobilized on controlled pore glass according to the recommended general procedure [25]; then, the immobilized enzyme was packed into a PTFE reactor of 2 cm × 1.0 mm I.D. In order to study the influence of variables affecting the enzymatic hydrolysis of cholesteryl esters by cholesterol esterase, a pool serum sample solution diluted five times with 0.15 mol/l NaCl in order to lower the excessively high concentration of total cholesterol was used. The diluted serum (0.1 ml of sample plus 0.4 ml of 0.15 mol/l NaCl) was introduced in the continuous system and merged with a stream of 6 mmol/l sodium cholate in 50 mmol/l buffer at pH 8. The serum–sodium cholate–buffer mixture was continuously circulated through an injection valve (IV_1 in Fig. 1) and only 120 μl was again injected into a buffer solution at pH 8 circulated at 0.5 ml/min. This injection valve was included in the flow manifold in order to avoid passing the whole serum sample through the immobilized enzyme and hence lengthen its lifetime, as well as to flush the serum sample with the buffer in order to make the flow

module ready for a new analysis. An injected volume of 120 μl was selected from those assayed (25–200 μl) as it ensured minimal dispersion of the sample in the PIPES carrier and a large sample zone for subsequent passage through IV_2 giving rise to reproducible results. The immobilized enzyme reactor (IMER) was immersed in a water bath at 40°C, and free cholesterol was extracted with petroleum ether (an additional ethanol stream was required to disrupt the lipoprotein micelles). Experimental variables were optimized by collecting the organic extract, containing the cholesterol, from the T-separator in several 0.2-ml fractions for 1 min and then injecting 2-μl aliquots of the fractions manually into the chromatograph by means of a syringe. The fraction used to obtain the analytical results was that yielding the highest signal in terms of peak area.

Three buffers at pH 8.0 were assayed, namely: potassium phosphate, Tris and PIPES, of which PIPES was selected because it resulted in a peak area that was ca. 2 times greater than that provided by the other two. The effect of the serum sample pH on the enzymatic hydrolysis of cholesterol esters was thus studied by using PIPES buffer at a pH of 6.5–8.5; the optimum pH range was found to be 7.5–8.3. A buffer solution of pH 8 was thus selected to adjust the pH of the serum sample. The effect of the concentration of PIPES buffer (containing 6 mmol/l sodium cholate) was studied over the range 10–250 mmol/l. Above 30 mmol/l, the signal remained virtually constant, so a 50 mmol/l solution of PIPES buffer at pH 8 was chosen.

Conventional methods use surfactants for stabilizing micelles and facilitate enzymatic hydrolysis [12–17]. We assayed the two most frequently used surfactants, Triton X-100 and sodium cholate, at a concentration of 6 mmol/l in the buffer solution. The results obtained in terms of peak area for cholesterol were better (1.5 times) for sodium cholate than for Triton X-100, so the former was selected. The concentration of sodium cholate added to the PIPES buffer was varied between 0 and 10 mmol/l. The chromatographic signal for cholesterol increased with the sodium cholate concentration up to 5 mmol/l. Above 7 mmol/l the analytical signal decreased slightly, however, as the likely result of rupture of the lipoprotein micelles by ethanol being hindered at high surfactant concentrations. For further experiments we thus used a sodium cholate concentration in the buffer of 6 mmol/l.

The flow-rate of the serum sample used for determining total cholesterol was identical with that of the serum sample for determining the HDL-C fraction, viz. 0.5 ml/min. The flow-rate of the stream carrying sodium cholate plus PIPES buffer was maintained constant at 0.5 ml/min so that the sodium cholate and PIPES concentration was the optimal. Maintaining a 50 mmol/l concentration of PIPES at pH 8.0 for the enzymatic hydrolysis of cholesterol was critical as the buffer acted as the carrier for the injected sample. For this purpose, we assayed buffer flow-rates between 0.1 and 0.6 ml/min. The signal increased initially as a result of the analyte dispersion in the buffer carrier decreasing at high flow-rates; however, above 0.55 ml/min, the signal started to decrease because the residence time of the sample in the IMER reactor was also decreased, so the enzymatic hydrolysis of cholesterol was incomplete. Between 0.45 and 0.55 ml/min, a steady state was roughly reached because the residence time of the sample in the IMER was quite satisfactory and any effects in the opposite direction to those described above were offset during the interval; therefore, a flow-rate of 0.5 ml/min was selected as optimal. The influence of the length of the immobilized enzyme reactor on the enzymatic hydrolysis of cholesteryl esters was studied over the range 0–5 cm. As can be seen in Fig. 3, a small analytical signal was initially obtained in the absence of the IMER reactor that corresponded to free cholesterol in the serum (about 30% of total cholesterol) [1]. Then, the signal increased with increasing length of the IMER up to 2 cm, above which it remained virtually constant. An IMER length of 3 cm was thus selected, which was adequate for complete hydrolysis of the cholesteryl esters. Finally, the effect of temperature on the efficiency of enzymatic hydrolysis was studied over the range 25–50°C. As can be seen in Fig. 4, the analytical signal increased with increasing

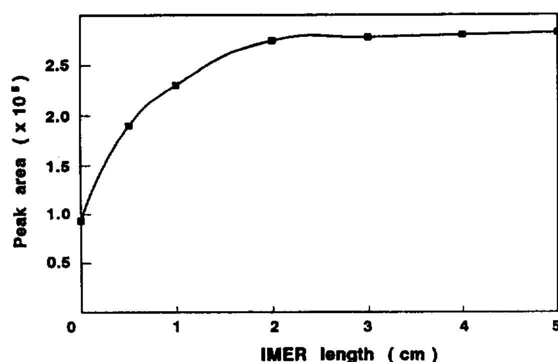

Fig. 3. Influence of the length of immobilized enzyme reactor (IMER) on the enzymatic hydrolysis of cholesteryl esters.

temperature up to 40°C in the IMER. Above 43°C, however, the signal started to diminish, probably through a decreased activity of the immobilized enzyme. The IMER was heated at 42°C in a thermostated water bath. At the end of each daily session, the IMER was flushed with 0.1 mol/l potassium phosphate, the end connectors were shut off to keep the phosphate solution in the reactor and the IMER was stored at 4°C; under these refrigerated conditions, the IMER remained active for at least three months.

Two extractants for cholesterol were assayed, viz. petroleum ether and *n*-hexane, of which the former was selected because it provided an analytical signal that was three times greater than that obtained with the latter. The effect of the flow-rate of petroleum ether was studied over the range 0.05–0.7 ml/min. The peak area increased with decreasing flow-rate as a result of the increased preconcentration ratio (aqueous-to-organic phase). An extractant flow-rate of 0.25 ml/min was thus chosen as a compromise between adequate extraction and reproducibility. Mixing of the stream from the IMER with the petroleum ether gave rise to an emulsion that hindered separation of the organic phase in the phase separator. In order to disrupt the micelles forming the emulsion, several reagents were assayed (viz. ethanol, 1,4-dioxane, ethyl acetate, ethyl ether, 2-propanol and methanol); they were placed in a stream that was merged with the petroleum ether stream. The best results were obtained by using ethanol, which was therefore selected. The effect of the flow-rate of ethanol on micelles rupturing lipoproteins was studied over the range 0.05–0.6 ml/min. Above 0.2 ml/min, rupture was maximal. In order to simplify the flow manifold, the streams carrying petroleum ether and ethanol were merged in one at 0.5 ml/min; thus, an extractant consisting of petroleum ether–ethanol (1:1) provided the same result as the two separate streams. The length of the coil located after the extractant line (see Fig. 1) was critical for efficient rupture of the lipoprotein micelles; a coil of 200 cm was required for this purpose and efficient extraction of cholesterol in the ether phase.

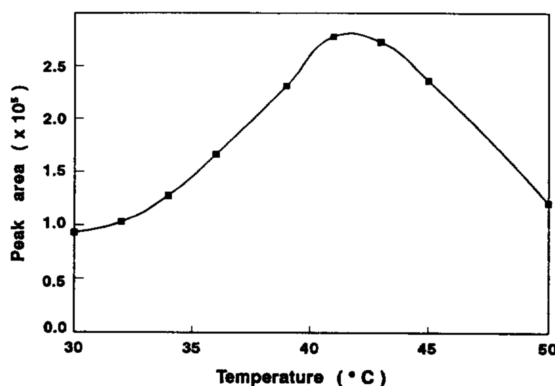

Fig. 4. Effect of temperature on the efficiency of enzymatic hydrolysis of cholesteryl esters.

Use of a membrane phase separator (Fluoropore membranes) [24] proved unsatisfactory as the membrane was clogged by particles from the circulating solution, as a result of the turbidity produced when the ethanol stream was inserted; the effect was especially noticeable for highly lipidemic serum samples. Thus, we chose a T-shaped glass-PTFE separator instead.

3.3. Interface between the continuous system and the gas chromatograph

The interface between the continuous system module and the gas chromatograph was an injection valve (with slight modifications for its present purpose), similar to that used elsewhere to couple an extraction unit to a gas chromato-

graph [24]. The injected volume of injection valve IV_2 was 5 μl; the valve was constructed from PTFE tubing and connected to the instrument via a 25 cm × 0.3 mm I.D. PTFE tube furnished with a needle at the end that was directly inserted into the septum of the injection port of the chromatograph. The flow-rate of the carrier gas was varied in order to reduce adsorption of cholesterol in the loop and connection tube valve port, as well as to improve chromatographic resolution of the peaks. For this purpose, the total carrier gas flow-rate was varied between 10 and 45 ml/min. An overall gas flow-rate of 37.8 ml/min (flow-rates through the valve and injection port 27.0 and 10.8 ml/min, respectively) was selected as optimal.

3.4. Determination of total cholesterol and HDL cholesterol

Calibrating the enzymatic determination of cholesterol with aqueous or organic solutions of cholesterol is made difficult by the low solubility of cholesterol. Calibration with a correctly labelled serum pool appears to be the best way to ensure the highest possible accuracy with the enzymatic method [12]. A Roche control serum with a certified total cholesterol content of 319 ± 17 mg/dl was used to prepare a series of samples that were diluted to a final volume of 0.5 ml with 0.15 *M* NaCl. A sterol not occurring in serum was selected as internal standard (stigmasterol); thus a solution containing 40 mg/l of stigmasterol in petroleum ether–ethanol (1:1) was used as extractant for cholesterol in the continuous system. The standard calibration graph ($y = 0.0096x - 0.0120$) obtained by plotting the analyte-to-internal standard peak area ratio (y) against the cholesterol concentration (x, mg/dl) was linear throughout the tested ranged, viz. 10–100 mg/dl. The correlation coefficient (r) for the linear regression is 0.998. The precision (expressed as the relative standard deviation), checked on eleven diluted control serum samples containing 40.5 mg/dl of total cholesterol, was 3.3%. The same Roche control serum, with a certified HDL-C content of 90 ± 9 mg/dl was also employed to determine the precision of the method in the determination of HDL-C; for this purpose, eleven diluted samples containing 59.5 mg/dl of HDL-C and the precipitating reagents were analysed. The precision thus obtained was 4.1%.

3.5. Validation of the proposed method

The proposed method was validated by analyzing a "Roche control serum N (equine)" with certified HDL-C (45 ± 8 mg/dl) and total cholesterol (77 ± 14 mg/dl) contents. For the HDL-C determination, 0.5 ml of control serum (pH 7.5) plus 187.5 μl of precipitating reagent solution (150 μl of 40 g/l sodium phosphotungstate and 37.5 of 2 mol/l magnesium chloride) was introduced into the automatic system. Total cholesterol in the control serum was determined by introducing a sample solution containing 0.3 ml of control serum plus 0.2 ml of 0.15 *M* NaCl. Each sample was individually prepared and analysed five times to obtain a mean value and standard deviation. The HDL-C and total cholesterol contents obtained were 45.1 ± 1.7 and 74.7 ± 2.5 mg/dl, respectively, which are consistent with the certified contents.

3.6. Application to human serum

The proposed method was applied to the determination of total cholesterol and HDL-C in human serum by using samples from donors with a negative serological test. For total cholesterol, a sample containing 0.1 ml of serum plus 0.4 ml of 0.15 *M* NaCl was introduced into the proposed system. HDL-C was determined by introducing a sample containing 0.5 ml of serum (pH 7.5) plus 187.5 μl of precipitating reagent (150 μl of 40 g/l sodium phosphotungstate and 37.5 μl of 2 mol/l magnesium chloride). The results obtained in triplicate analyses of the ten donor serum samples and their standard deviations are given in Table 1. The total cholesterol concentrations varied between 143.7 and 278.2 mg/dl; however, the HDL-C concentrations in the analysed serum samples varied over a narrower range (42.8–56.9 mg/dl). Fig. 5 shows typical chromatograms for total cholesterol (A) in a

Table 1
Determination of total cholesterol and HDL cholesterol in human serum

Serum sample	Total cholesterol (mg/dl)[a]	HDL cholesterol (mg/dl)[a]
1	278.2 ± 0.6	46.8 ± 0.3
2	163.3 ± 1.5	49.0 ± 0.3
3	186.9 ± 1.8	42.8 ± 0.1
4	174.1 ± 1.1	49.2 ± 1.0
5	231.2 ± 0.3	56.9 ± 0.1
6	150.2 ± 1.5	44.8 ± 0.1
7	165.1 ± 1.3	47.8 ± 0.1
8	268.9 ± 1.5	51.5 ± 0.4
9	143.7 ± 1.8	50.3 ± 0.9
10	145.8 ± 1.0	47.4 ± 0.2

[a] Mean ± standard deviation ($n = 3$).

serum sample diluted five times with 0.15 mol/l NaCl, and HDL-C (B) in the same sample.

4. Conclusions

Flow-injection methodology had only been applied so far to the determination of total cholesterol by using immobilized enzymes and amperometric [14,15] or photometric [16,17] detection. Conventional methods for HDL-C determination generally involve tedious manual procedures using polyanions with divalent metals to precipitate very-low- and low-density lipoproteins; after centrifugation, HDL-C is quantitated by enzymatic assay of cholesterol in the supernatant solution, followed by derivatization with various reagents (e.g. 4-aminophenazone, 4-aminoantipyrine) for photometric monitoring [9,12]. Such automatic systems as the Hitachi 705 (Boehringer, Mannheim, Germany), Technicon RA 1000 (Technicon Instruments, Tarrytown, NY, USA), Cobas Bio analyzer (Hoffmann-La Roche, Basel, Switzerland) and Reflotron HDL Cholesterol (Boehringer) are now commercially available for use by clinical laboratories [26]. The proposed system permits the sequential determination of total cholesterol and HDL-C by gas chromatography; in addition, the module can be fitted to a photometer by introducing colorimetric reagents rather than the organic extractant into the flow system. However, the use of a gas–liquid chromatograph coupled on-line to the proposed module makes it more selective than alternative photometric procedures, which are disturbed by the presence of some drugs given similar colours as cholesterol in the serum sample [17,26].

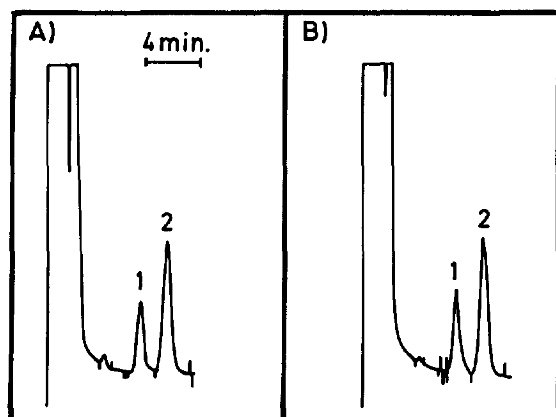

Fig. 5. Gas chromatograms for total cholesterol in a diluted serum sample (A), and HDL-C in an undiluted serum sample (B): 1 = cholesterol; 2 = internal standard (stigmasterol).

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