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Quantitative detection method of triglycerides in serum lipoproteins and serum-free glycerol by high-performance liquid chromatography

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Abstract

We have developed a simple and reliable method for quantitative detection of triglycerides (TG) in serum lipoproteins and serum-free glycerol (FG) by high-performance liquid chromatography (HPLC). After separation of serum constituents using a new gel-permeation column (TSK gel Lipopropak XL, Tosoh) and a new eluent (TSK eluent LP-2, Tosoh), TG and FG were detected by on-line reaction using a modified reagent which contained glycerol kinase, glycerol-3-phosphate oxidase and lipoprotein lipase. HPLC patterns showed five peaks corresponding to chylomicrons, very-low-density, low-density, high-density lipoproteins and FG. Absolute concentrations of TG in each lipoprotein fraction and serum FG were calculated from the corresponding peak areas using standard FG as a calibrator. Due to its very high sensitivity of peak detection, this method has become desirable for the analyses of lipoproteins of very low concentrations such as in cell culture systems. This technique will contribute to a better understanding of lipoprotein TG and serum FG distribution in human and nonhuman subjects.

Keywords: Triglycerides; Lipoproteins; Free glycerol

1. Introduction

It is well known that serum low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol are positive and negative risk factors for the atherosclerotic diseases, respectively [1]. Therefore, measurement of cholesterol in major serum lipoproteins has become an important routine practice [2,3]. There have been several reports [4–7] that high serum TG is an independent risk factor for atherosclerosis and therefore, clinical significance of serum TG in major lipoprotein classes is now being assessed. However, no practical method for measurement of TG in major lipoprotein classes is available at present.

In 1980, we successfully applied a high-performance liquid chromatography (HPLC) technique to lipoprotein analysis [8]. Using the HPLC technique, we have reported selective detection of TG [9], cholesterol [10] and phospholipids [11] by on-line enzymatic reaction. In our earlier technique, however, we could not eliminate nonspecific adsorption

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of lipoproteins to TSK-gel materials (TSK gel SW and PW type, Tosoh). In 1994, improved gel permeation columns (TSK gel Lipopropak and Lipopropak XL, Tosoh) and especially prepared eluents (TSK eluent LP-1 and LP-2, Tosoh) became available for lipoprotein analysis. We have reported a simple, accurate and rapid method for cholesterol measurement in major lipoprotein classes by HPLC using the new columns (TSK gel Lipopropak, Lipopropak XL) and eluent (TSK eluent LP-1) followed by enzymatic cholesterol detection [12–16]. Since the TSK eluent LP-1 is limited only to cholesterol determination, we tried to determine TG in major lipoproteins using TSK eluent LP-2 instead of LP-1 and successfully established an quantitative method of TG and FG using the HPLC system.

In this paper, we examined the optimum reaction conditions for on-line detection of TG and FG. The accuracy of this method was examined by comparison of total TG values with the chemical autoanalyzer and linearity test of standard lipoproteins and FG. Moreover, examples of the application of this technique for lipoprotein metabolism are presented using an animal model.

2. Experimental

2.1. Reagents

Total cholesterol (TC) and TG of sera and standard lipoproteins were determined enzymatically by using Determiner L TC and Determiner L TG (Kyowa Medex). All measurements were performed with a chemical autoanalyzer (Hitachi 7150).

For the detection of TG and FG in HPLC method, we used kit reagents for TG determination by chemical autoanalyzer (Determiner L TG, Kyowa Medex) and its modified reagents with higher amounts of enzyme components were used. This reagent kit comprises reagent 1 (R1) and reagent 2 (R2). R1 contains glycerol kinase (GK, 1000 U/l), glycerol-3-phosphate oxidase (GPO, 8000 U/l), adenosine-triphosphate (ATP, 4.5 mmol/l), *N*-ethyl-*N*-(3-methylphenyl)-*N′*-succinyl-ethylendiamine (1.1 mmol/l), ascorbic acid oxidase (3000 U/l), $MgSO_4$ (2.0 mmol/l), 50 mmol/l 3-(*N*-morpholino) propanesulphonic acid (MOPS) buffer (pH 6.25), detergent and stabilizer. R2 contains lipoprotein lipase (LPL, 2000 U/l), peroxidase (20 000 U/l), 4-aminoantipyrine (2.5 mmol/l), $MgSO_4$ (4.0 mmol/l), 10 mmol/l MOPS buffer (pH 6.25), detergents and stabilizer.

Modified reagents were prepared as follows. The concentration of LPL was increased thus, 500, 2000, 6000, 9000, 12 000, 15 000, 18 000, 24 000 U/l by adding lyophilized LPL to commercial R2. The concentration of GK and GPO was increased to three times and six times of commercial R1 by adding respective lyophilized enzymes to the commercial R1.

2.2. HPLC

The HPLC system included a high-speed chemical derivatization chromatograph (CCP and 8010, Tosoh) equipped with a gel permeation column (TSK gel Lipopropak XL, 300×7.8 mm I.D., Tosoh). Ten μl of whole serum or plasma and a diluted (0.15 *M* NaCl, 20 m*M* Hepes, pH 7.5) solution of standard lipoprotein or FG were applied to the column and eluted by eluent (TSK eluent LP-2, Tosoh) at a flow-rate of 0.60 ml/min. The column effluent was mixed with reagent 1 (R1, see above) at a flow-rate of 0.225 ml/min and reagent 2 (R2) at a flow-rate of 0.075 ml/min. The mixed solution was passed through a reaction tube (Teflon, 0.4 mm in diameter and 2.5 m, 7.5 m, 10 m, 12.5 m, 15 m and 30 m in length) in a thermostated water bath at 25–45°C. The amount of a colored substance produced was measured at 550 nm by an ultraviolet and visible range detector (UV-8010, Tosoh). The absolute TG and FG concentrations were calculated from the peak area using a standard aqueous solution of FG (2.26 mmol/l of FG which was equivalent toTG of 2.26 mmol/l or 200 mg/dl as triolein) as a calibrator.

2.3. Test tube reaction

A 24-μl sample was added to a mixed solution of 2.4 ml R1 and 0.8 ml R2. Both R1 and R2 were diluted 3 times with the eluent (TSK eluent LP-2). The colored substance produced in a test tube reaction at 37°C was determined by a spectrophotometer (U-3300, Hitachi) at a reaction time of 0 to 570 s.

2.4. Samples

We used serum from a hyperlipidaemia (subject 1, TC=4.89 mmol/l, TG=1.92 mmol/l) for a test-tube reaction, and serum from a patient with LPL deficiency (subject 2, TC=4.58 mmol/l, TG=8.67 mmol/l) and a healthy female (subject 3, TC=3.46 mmol/l, TG=0.407 mmol/l) for detailed examination to determine the optimum reaction condition for HPLC method. Sera from 100 subjects (TC= 5.52±1.96 mmol/l, 1.9~11.87 mmol/l, TG= 2.44±2.41 mmol/l, 0.35~10.5 mmol/l) were used for comparison of TG values by HPLC method and those by chemical autoanalyzer.

Standard lipoproteins, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Chemicon International) were used for peak identification in HPLC patterns corresponding to lipoprotein fractions in test samples as well as for the linearity test. Plasma samples collected from a normal rabbit (male, five month) and a Watanabe heritable hyperlipidaemic (WHHL) rabbit (hetero, male, five month) were analyzed before and after intravenous injection of glucose (0.6 g of glucose per kg of body weight). Supernatant fractions collected from conditioned culture medium of normal rabbit hepatocyte were analyzed.

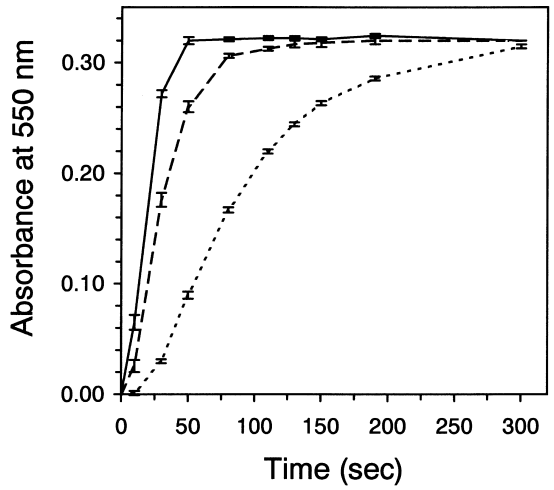

Fig. 1. Effect of glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO) concentration on enzymatic reaction. A 24 μl volume of human serum (subject 1) was added to a mixed solution of 2.4 ml R1 and 0.8 ml R2, both of which were diluted 3 times with eluent buffer (TSK eluent LP-2). R2 contained six times LPL compared with the commercial kit and R1 contained an equal amount (···), three times (- - -) and six times (———) GK and GPO of the commercial kit concentration. The reaction temperature was 37°C. The average value (n=3) of absorbance at 550 nm was plotted against the reaction time.

3. Results

3.1. Examination of optimal GK and GPO concentration

We examined an optimum concentration of GK and GPO in R1 used for on-line reaction in HPLC method. In Fig. 1, the time profiles of reaction in the test tube using three-times diluted reagents with TSK eluent LP-2 and serum from subject 1 are presented. To eliminate the effect of LPL concentration, we used R2 containing 12 000 U/l of LPL (six times of commercial LPL). R1 containing three times GK and GPO concentrations showed remarkably enhanced reaction compared to commercial R1, but no significant further enhancement was observed with six times GK and GPO concentration in the reaction time over 120 s (Fig. 1). Based on the results of this experiment, R1 containing three times GK and GPO of commercial reagent was considered to be suitable for the HPLC method.

3.2. HPLC elution patterns

In Fig. 2, the elution profile monitored by the enzymatic reaction under optimized experimental conditions as described later are shown for serum samples (subjects 2 and 3) and standard lipoproteins. HPLC patterns gave five peaks, and comparison of the elution patterns with standard lipoproteins revealed that peak 2, peak 3 and peak 4 corresponded to VLDL, LDL and HDL, respectively. Peak 1, at the void volume observed with a sample from a patient with LPL deficiency and peak 5 observed with subject 2 and 3 were confirmed to correspond to chylomicrons (CM) and FG, respectively, by comparing the patterns of ascitic CM and FG standard (pattern not shown).

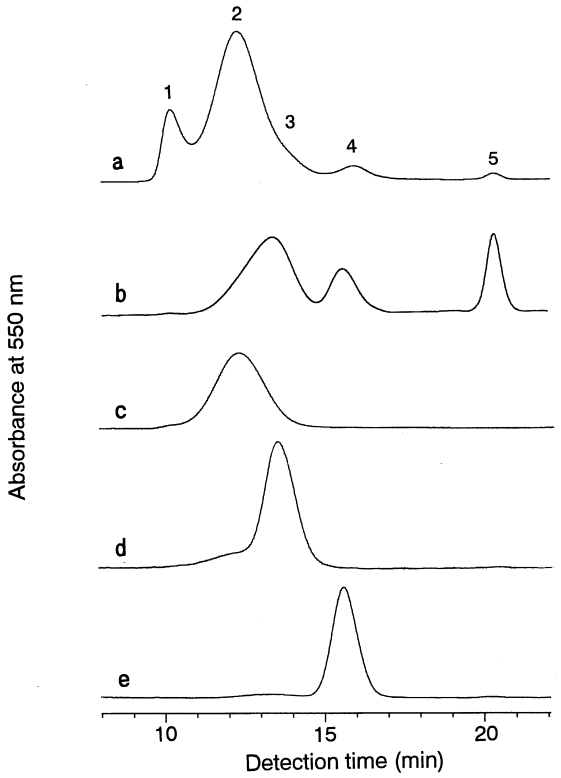

Fig. 2. Elution patterns of TG and FG for human sera and standard lipoproteins. Samples: 10 μl of diluted solution (0.15 *M* NaCl, 20 m*M* Hepes, pH 7.5) of human serum or a standard lipoprotein (Chemicon International). a, Subject 2 (LPL deficiency), ×10; b, subject 3 (healthy female), ×1; c, VLDL, ×50; d, LDL, ×2; e, HDL, ×10. Peak 1, chylomicrons; peak 2, VLDL; peak 3, LDL; peak 4, HDL; peak 5, FG. HPLC conditions: column, TSK gel Lipopropak XL (300×7.8 mm I.D.); eluent, TSK eluent LP-2 (flow-rate=0.6 ml/min); enzyme reagent, R1 (3000 U/l of GK and 24 000 U/l of GPO, flow-rate=0.225 ml/min) and R2 (12 000 U/l of LPL, flow-rate=0.075 ml/min); detector (A550), 100 mV at full scale.

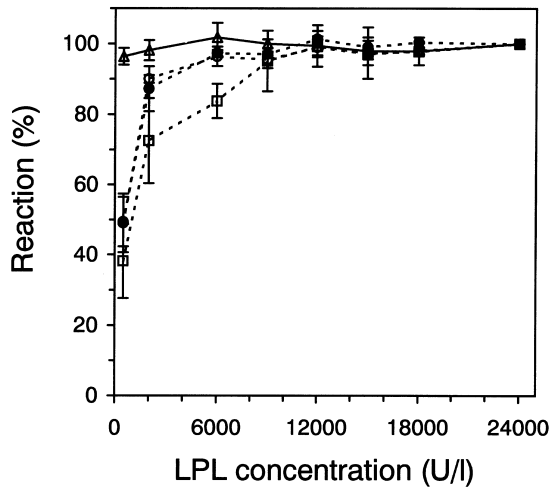

Fig. 3. Effect of concentration of LPL on enzymatic reaction. Samples: 10 μl of two-times diluted serum (subject 2). HPLC analysis was done under the optimized experimental conditions (as in Fig. 2) except for R2 with different LPL concentrations. Reaction % (calculated based on 100% at LPL concentration of 24 000 U/l) for each peak (1, □; 2+3, ●; 4, ○; 5, △) was plotted against LPL concentration.

3.3. Examination of optimized conditions for on-line enzymatic reaction

3.3.1. LPL concentration

We examined the effect of LPL concentration on the reaction of TG in each lipoprotein fraction and FG with the HPLC by using modified R2 with various amounts of LPL (500 to 24 000 U/l) and optimized R1 in a reaction tube (10 m in length) at 37°C. Results obtained for subject 2 are presented in Fig. 3. Reaction percentages were calculated by dividing each peak area (peaks 1–5 in Fig. 2) by the corresponding peak area at maximum LPL concentration (24 000 U/l). Reaction % for peak 5 (FG) was independent of LPL concentration, but that of peak 1 (CM) was strongly dependent on LPL concentration. A good reaction for all peaks was obtained in R1 containing more than 12 000 U/l.

3.3.2. Reaction time

The reaction time in HPLC method was changed from 0.45 min to 5.34 min using Teflon tube of 2.5 m to 30 m in length (0.4 mm I.D.). The relation of reaction % for each peak to reaction time using optimized R1 and R2 at 37°C showed that 1.8 min of reaction time (tube of 10 m in length) was suitable for all peaks as shown in Fig. 4.

3.3.3. Reaction temperature

Optimum reaction temperature in a reaction tube (10 m in length) in the HPLC system was examined using optimized R1 and R2. As shown in Fig. 5, all cases (subjects 1, 2 and 3) showed maximum reaction at 37°C.

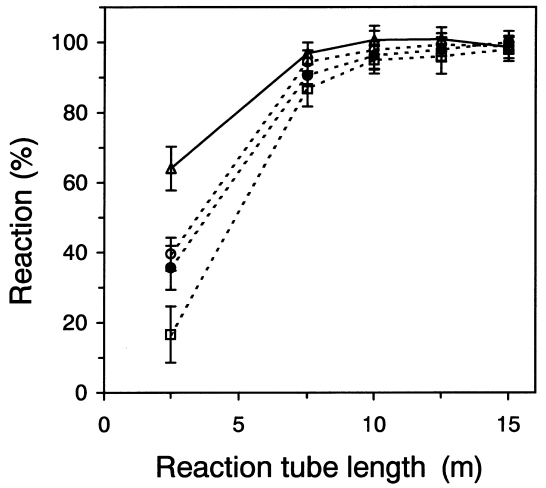

Fig. 4. Effect of reaction time on enzymatic reaction. Samples and experimental conditions were the same as in Fig. 2 except for reaction tube with varying length, from 2.5 m to 30 m. Reaction % for each peak (1, □; 2+3, ●; 4, ○; 5, △) based on 100% at a tube length of 30 m was plotted against tube length.

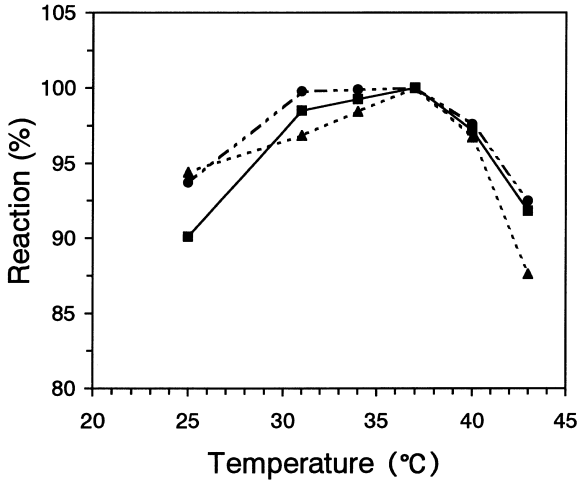

Fig. 5. Effect of reaction temperature on enzymatic reaction. Samples: 10 μl of two-times diluted sera (subject 1, ●; subject 2, ■; subject 3, ▲). HPLC analysis was done under the optimized conditions (as in Fig. 2) except for temperature. Reaction % was calculated from total peak areas based on 100% at maximum peak area for individual subjects.

3.4. Linearity and detection limit

We examined the relation between peak area and concentration for a standard of VLDL and FG under the optimized experimental conditions (Fig. 6). Linear relations were obtained in the concentration range 0.2 mmol/l to 2 mmol/l for both standards. Detection limits for VLDL peak and FG peak were 1.5 μmol/l and 0.3 μmol/l when 10 μl of standards were applied to the HPLC system.

3.5. Precision data

Within-day and between-day (consecutive five days analyses) precision for TG in lipoprotein fractions and serum FG were examined for hypertriglyceridaemic and normotriglyceridaemic subjects

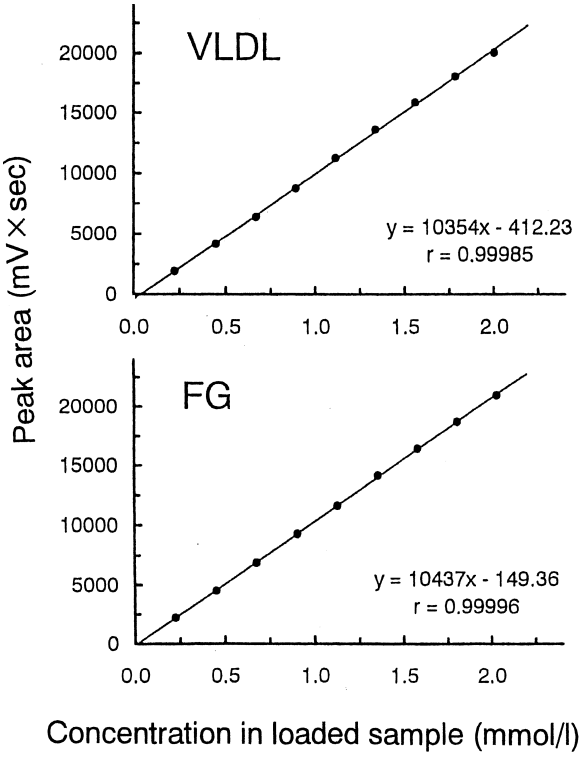

Fig. 6. Relation between peak area and concentration in loaded sample. Samples: 10 μl of diluted solution (0.15 *M* NaCl, 20 m*M* Hepes, pH 7.5) of standard samples. VLDL (upper) and FG (lower). HPLC conditions as in Fig. 2.

Table 1
Precision of TG in lipoprotein fractions and serum FG by HPLC method

			Total	Peak 1 CM	Peak 2+3 VLDL+LDL	Peak 4 HDL	Peak 5 FG
Within-day (n=5)	subject 2	AVG, mmol/l	7.953	1.385	6.141	0.427	0.054
		S.D., mmol/l	0.121	0.039	0.129	0.009	0.002
		C.V., %	1.52	2.82	2.10	2.13	3.72
	subject 3	AVG, mmol/l	0.390	–	0.295	0.095	0.102
		S.D., mmol/l	0.006	–	0.005	0.002	0.003
		C.V., %	1.44	–	1.59	1.64	2.83
Between-day (n=5)	subject 2	AVG, mmol/l	7.870	1.371	6.080	0.419	0.060
		S.D., mmol/l	0.121	0.041	0.136	0.009	0.002
		C.V., %	1.53	3.02	2.23	2.18	4.06
	subject 3	AVG, mmol/l	0.388	–	0.292	0.096	0.105
		S.D., mmol/l	0.006	–	0.005	0.001	0.003
		C.V., %	1.48	–	1.57	1.22	2.99

Subject 2, LPL deficiency; subject 3, healthy female.
–: Peak not detected.

(Table 1). Coefficient of variation values for the HPLC method were between 1.22% and 4.06%.

3.6. Correlation between total TG by HPLC and by chemical autoanalyzer

As for human subjects (n=100), total TG concentration was calculated from total peak areas (peak 1–4) using standard FG (2.26 mmol/l) as a calibrator. The results were compared with those measured by chemical autoanalyzer (Fig. 7) and summarized for three groups classified by the TG values (Table 2). Very strong correlations were obtained between the two methods and paired t-test showed no significant difference except for the group with TG values less than 1.70 mmol/l ($P<0.01$).

3.7. Application for studying lipoprotein metabolism

We applied the HPLC method to analyze the change of lipoprotein profiles induced by glucose injection to a normal rabbit and to a WHHL rabbit (hetero). Typical examples of HPLC patterns are shown in Fig. 8. For both cases, increases in larger particle-size fractions in peak 2 corresponding to VLDL were observed after injection of glucose. Increases in FG peaks were clearly observed in the patterns of upper case after injection of glucose.

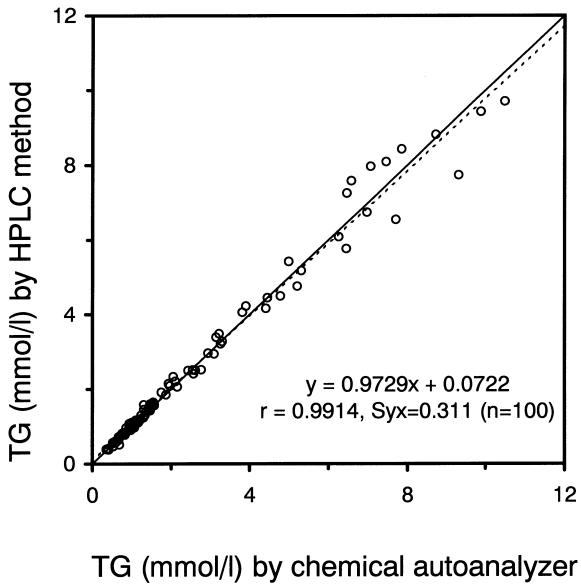

Fig. 7. Comparison of TG measured by HPLC with those by chemical autoanalyzer for 100 human sera. A 10 μl volume of serum was applied to the HPLC system and the TG concentration was determined from total peak areas (peak 1–4) using a standard FG as a calibrator. Linear regression and ideal correlation ($y=x$) are shown in dotted line and solid line, respectively.

Table 2
Comparison of triglycerides values between HPLC method (*y*) and chemical autoanalyzer (*x*)

Group	TG levels mmol/l	*n*	Mean±S.D. *y*, mmol/l	Mean±S.D. *x*, mmol/l	Slope	*y*-intercept	*Syx*	*r*
A	<1.70	61	1.01±0.36[a]	0.99±0.34	1.040	−0.019	0.068	0.9825
B	1.70–4.40	20	2.73±0.69	2.68±0.65	1.028	−0.020	0.173	0.9695
C	>4.40	19	6.77±1.75	6.86±1.82	0.887	0.681	0.689	0.9235
All		100	2.45±2.36	2.44±2.41	0.973	0.072	0.311	0.9914

[a] $P<0.01$.

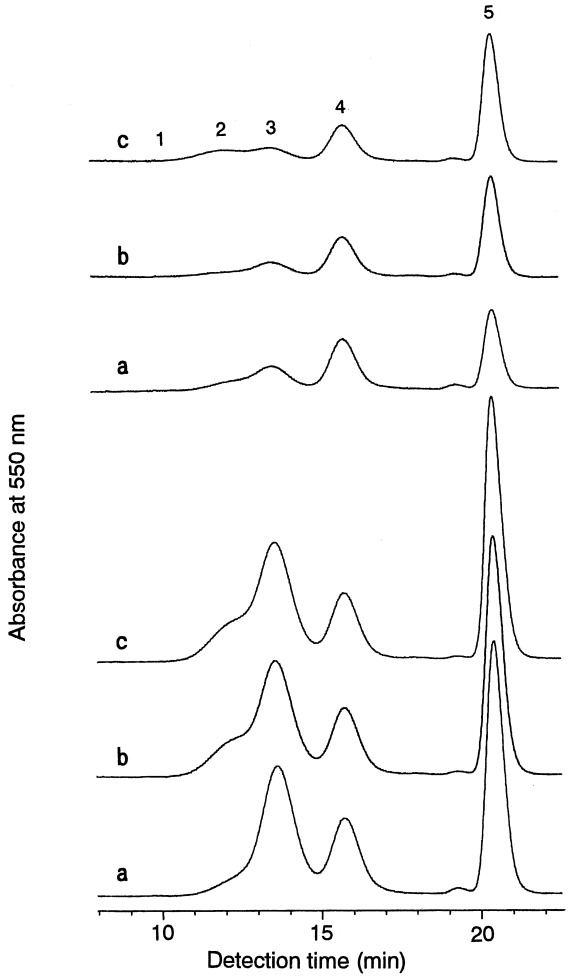

Fig. 8. Changes in HPLC patterns induced by injection of glucose to normal rabbit (upper) and WHHL rabbit (lower). Samples: 10 μl of plasma collected before injection (a), 45 min (b), and 120 min (c) after injection of glucose. HPLC conditions and peaks as in Fig. 2. The HPLC patterns are shown at 100 mV (full scale).

We also applied this method for the analyses of lipoproteins secreted by normal rabbit hepatocyte in cell culture systems (Fig. 9). HPLC patterns obtained by applying 250 μl of supernatant of conditioned medium revealed that the main lipoprotein fraction was VLDL which increased according to incubation

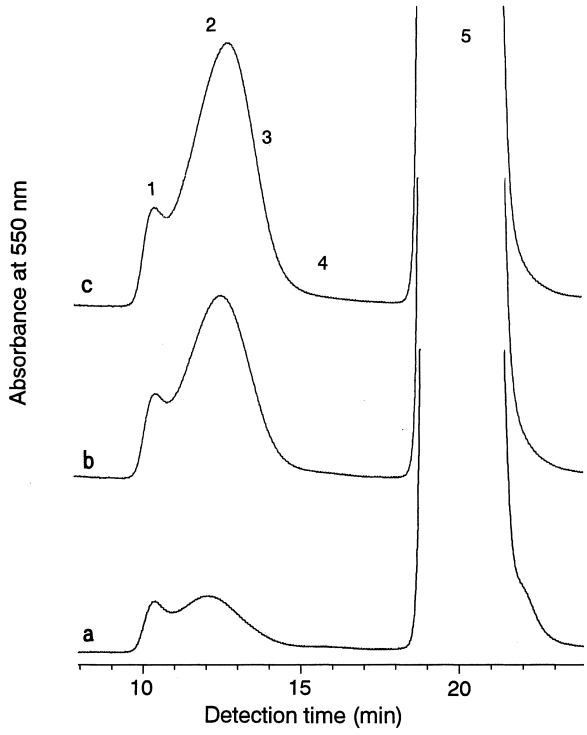

Fig. 9. HPLC patterns for lipoproteins secreted from normal rabbit hepatocytes in a cell culture system. Samples: 250 μl of supernatant of conditioned medium incubated for 24 h (a), 48 h (b) and 72 h (c). HPLC conditions and elution position of peaks as in Fig. 2. The HPLC patterns are shown at 60 mV (full scale).

time; lipoproteins corresponding to LDL and HDL were not detected. This system could provide reliable information about lipoproteins of very low concentration, less than 0.01 mmol/l (pattern a in Fig. 9).

4. Discussion

The determination of TG and cholesterol in serum lipoprotein fractions is very useful for the diagnosis of dyslipoproteinaemia and status of lipoprotein metabolism. TG determination in lipoprotein fractions has been carried out by ultracentrifugation [17], gel filtration chromatography [18] and electrophoresis [19]. However the former two methods are time consuming, requiring large amounts of test samples. The latter method is a semiquantitative approach.

In 1982, we reported a simple and rapid method for selective detection of triglycerides using enzymatic reaction after separation by high-performance gel permeation chromatography [9]. Although qualitative analysis for triglyceride distribution among lipoprotein classes could be obtained from small amounts of whole serum or plasma, the method lacked the quantification mainly because of nonspecific reactivity of glycerol oxidase [20] present in the enzymatic reagent kit for triglyceride determination. Currently, the methods for the determination of TG have been much improved [4]. In this study, we used a commercial reagent kit using GK and GPO [21] instead of glycerol oxidase. Additionally, we prepared a modified enzyme reagent containing increased amounts of the key enzymes, GK, GPO and LPL, in order to compensate for the dilution effect of enzyme reagents with eluent in HPLC system. We confirmed in the test tube and HPLC system that using an optimized reagent concentrations, containing three times GK and GPO and six times LPL of commercial reagent, the end point in enzymatic reaction was obtained at a reaction time of 1.8 min, 37°C.

With the HPLC method under optimized conditions, five peaks corresponding to different particle sizes were observed (Fig. 2). Peak 1, CM; peak 2, VLDL; peak 3, LDL; peak 4, HDL; peak 5, FG. TG and FG concentration corresponding to these peaks were calculated using standard FG as a calibrator. Within-day and between-day precision data for the present method were excellent (Table 1), and very good linearity with the same slope of the regression line was obtained for standard VLDL and FG (Fig. 6). Comparison of total TG values measured by the present method with those by the chemical autoanalyzer showed very good correlation for 100 sera samples with TG values from 0.35 to 10.5 mmol/l (Fig. 7, Table 2). These results showed that the present method using TSK eluent LP-2 proved to be very accurate and reliable for recovery or determination of lipoproteins and that the elimination of the nonspecific adsorption of lipoproteins to gel materials was confirmed.

Since the sensitivity of peak detection was markedly improved due to elimination of nonspecific adsorption, this method was found to be useful for the analyses of lipoproteins at very low concentration such as in hepatocyte culture systems (Fig. 9). By applying 10 μl of whole serum or plasma with the present method, accurate quantitative information about TG concentration in individual lipoprotein classes and serum FG can be obtained. As demonstrated by the glucose tolerance test in an animal model (Fig. 8), this method is a useful practical approach to study triglyceride metabolism in serum lipoproteins and serum FG following various tolerance tests or stimulation test. Additionally, with a combination of cholesterol detection HPLC system [12,13], changes in lipoprotein TG as well as in cholesterol can be clarified. We believe that such information will contribute to a better understanding of lipoprotein metabolism in human and nonhuman subjects.

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