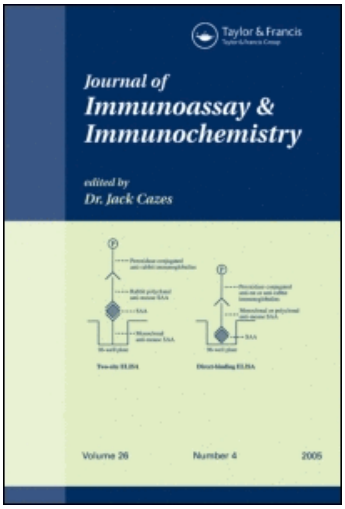

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Change in Immunoreactive Human Hepatic Triglyceride Lipase (HTGL) Mass and the Shelf-Life of the HTGL ELISA Kit in Long-Term Storage

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Abstract: Objectives of this work are to study changes in the immunoreactive HTGL mass during storage under various conditions. In addition, the shelf-life of the HTGL ELISA kit was confirmed. The immunological reactivity of HTGL in PHP stored in the liquid, frozen, or lyophilized state was monitored using purified human PHP-HTGL as the standard material. Furthermore, the long-term stability of the HTGL ELISA kit was ascertained. The immunoreactive HTGL mass in the lyophilized PHP maintained its initial immunological reactivity for at least 26 months at 4°C or lower. The other reagents included in the HTGL ELISA kit also have a long shelf-life when they are stored at 4°C or less. HTGL in PHP was stabilized by lyophilization and can be used as the standard material for HTGL ELISA; the HTGL ELISA kit has a long shelf-life, i.e., more than two years.

Keywords: HTGL, Immunoreactive mass, Immunological stability, Immunological activity, Shelf-life, ELISA

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INTRODUCTION

Hepatic triglyceride lipase (HTGL, EC 3.1.1.3) is a glycoprotein enzyme with a molecular weight of 65 kDa with 477 amino acid residues for the mature form of the protein; it is catalytically active in the monomeric form.[1] HTGL is synthesized and secreted by the hepatocytes and is transferred to the surface of hepatic endothelial cells, where it is believed to be anchored to heparan-sulfate proteoglycans and exerts physiological function.[2,3]

HTGL acts on the catabolism of plasma lipoproteins, such as intermediate-density lipoprotein (IDL) and high-density lipoproteins (HDL). HTGL works with an essential task in the conversion of IDL to low-density lipoprotein and HDL_2 to HDL_3 by hydrolyzing triglycerides in IDL and HDL_2.[2,3]

HTGL is usually present as a catalytically inactive enzyme, at a low concentration, in the circulation. On the other hand, an active type of HTGL is released from a heparan sulfate-like sugar chain, located on the surface of hepatic endothelial cells, into the circulation by intravenous administration of a certain amount of heparin (10–100 IU/kg of body weight). Post-heparin plasma (PHP) obtained in this way contains catalytically active-type HTGL and, therefore, is often used as a clinical sample for clinical investigation of HTGL. In general, diagnosis and identification of abnormalities of HTGL enzyme, such as HTGL deficiency[4,5] and low activities of HTGL in patients with certain liver diseases,[6] uremia,[7] or hypothyroidism[8] can be performed by measuring the enzymatic activity of catalytically active-type HTGL using PHP.[1,9] However, nowadays, methods used widely for measuring HTGL enzyme-catalyzed activity is based on the hydrolysis of a gum-arabic-emulsified, radiolabeled substrate[10–12] after inhibition of lipoprotein lipase (LPL, EC3.1.1.34) coexisting in PHP with 1 mol/L NaCl or with rabbit anti-human LPL polyclonal antibody.[1] Due to these laborious procedures with a long time requirement, the methods for the measurement of HTGL enzymatic activity have been thought to be unpractical and limited for a multi-sample assay.

We have recently succeeded in establishing a microplate-type ELISA, based on a direct two-step sandwich immunoassay, using a combination of two distinct types of monoclonal antibodies which recognize two different epitopes on the human HTGL molecule[13] to overcome this problem. This ELISA has made it possible to quantify the immunoreactive mass of HTGL enzyme protein.[9,13] HTGL protein, purified from human PHP,[1] was used as a reference material for developing this HTGL ELISA. In order to popularize this ELISA widely, it is essential to use an HTGL protein as a standard material which remains in a stable form for a long period. For this purpose, in the present study, we attempted to prepare lyophilized human PHP containing HTGL and evaluated the immunological stability of HTGL mass in this state. In addition, a long-term shelf-life of reagents such as immobilized anti-HTGL monoclonal antibody and HRP-labeled anti-HTGL monoclonal

antibody included in the HTGL ELISA kit was validated using an immunological reactivity to human HTGL protein as an indicator of stability for monitoring.

EXPERIMENTAL

Materials

HTGL protein was purified from human PHP according to the previously reported methods,[1] and the protein content was measured by BCA protein assay (BioRad, Sweden) using bovine serum albumin as a standard material. The purified PHP-HTGL protein was added to human plasma which was heat-treated for 60°C for 1 hour to eliminate the endogeneous HTGL immunoreactivity, and was then stored in liquid nitrogen. It was used as a control sample for the evaluation of the HTGL ELISA MARUPI Kit (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan).[13] The human PHP for the evaluation of the immunological activity of HTGL protein was purchased from Oct Co., Ltd. (Tokyo, Japan). According to the manufacturer's specification sheet, the human PHP was prepared by intravenous administration of sodium heparin (Novo Industry, A/S, Denmark), at a dose of 30 IU/kg of body weight, with subsequent centrifugation of the blood sample collected in tubes (Venoject, VT-070Na, TERUMO, Tokyo, Japan) containing Na_2-EDTA (1.5 mg/mL) 10 minutes after the injection of heparin. The PHP was stored in three states, i.e., liquid, frozen, and lyophilized. The immunoreactive HTGL mass concentration in these three states of PHP was measured by the HTGL ELISA kit using the purified human PHP-HTGL standardized each time for the periodic analysis of immunological stability of HTGL protein mass.

Storage of PHP Containing HTGL Protein

PHP was divided into some plastic vials (Iwaki, Tokyo, Japan) in a volume of 150 μL/vial and stored at 4°C or 25°C for 0, 2, 9, 24, 32, and 48 hours. Some of the plastic vials containing 150 μL of PHP were frozen at −20°C or −80°C for 2 months and kept in liquid nitrogen for 26 months. Additionally, PHP was divided into glass vials (Naigai Garasu Kogyo, Osaka, Japan) in a volume of 500 μL for lyophilization. These vials were subjected to preliminary freezing at −30°C for one hour, then to lyophilization for 18 hours at a vacuum level of 0.01 torr, using the lyophilizing machine ULVAC DF-03H (Nihon Sinku Gijutsu, Tokyo, Japan). After the process of lyophilization, the reduced pressure in the chamber of the machine was gradually normalized with nitrogen gas. Vials containing lyophilized PHP were capped and kept at −80, −20, 4, 25, or 37°C for 26 months at maximum.

Quantification of Immunoreactive HTGL Mass Concentration in PHP Stored at Different Conditions by the HTGL ELISA

Of the PHP stored in the liquid, frozen, or lyophilized forms, the frozen PHP was thawed and the lyophilized PHP was reconstituted with 500 μL of distilled water, then immunological activity of HTGL immunoreactive mass in these PHP preparations was measured by HTGL ELISA MARUPI kit using standard HTGL in lyophilized PHP in reference to the control sample which was stored frozen in liquid nitrogen. This HTGL ELISA kit was used according to the manufacturer's manual, as shown in Figure 1.

This ELISA was based on a direct sandwich-ELISA, using the combination of two distinct mouse anti-human HTGL monoclonal antibodies.[9,13] The diluted PHP preparation (20 μL) and 80 μL of an assay buffer (40 mmol/L phosphate buffer, pH 7.0, containing 0.15 mol/L NaCl, 0.3% Tween 20, 0.1% bovine serum albumin, 0.1% ProClin 150 and 0.8 IU of heparin) were dispensed into the wells of a microtiter plate coated with

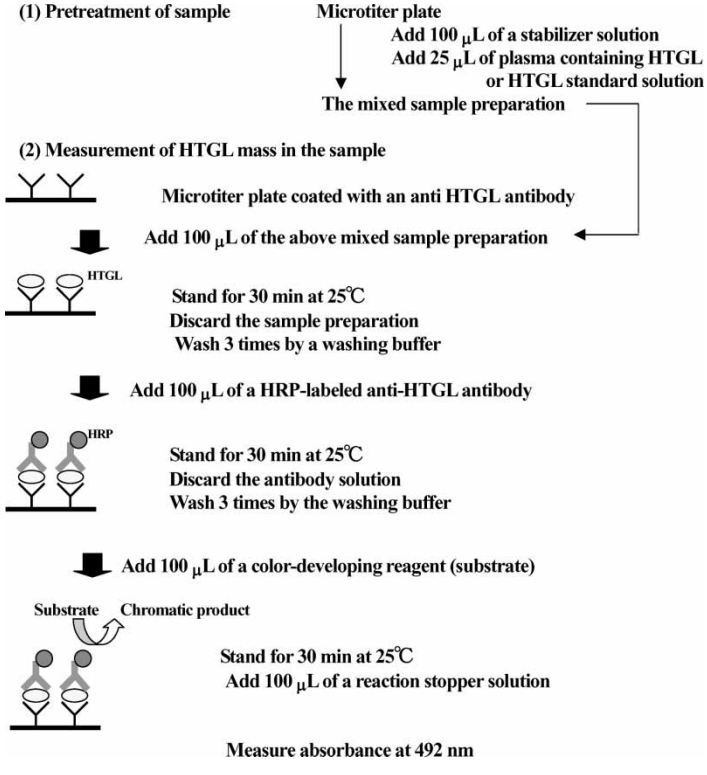

Figure 1. Assay procedure of the HTGL ELISA MARUPI kit. Assay system consists of (1) pretreatment of sample and (2) measurement of HTGL mass in the sample.

anti-human PHP-HTGL monoclonal antibody. The microtiter plate was incubated for 30 min at 25°C to allow the HTGL molecules to bind to the monoclonal antibody coated on the plate. The microtiter plate was washed three times with 5 mmol/L phosphate buffer, pH 7.0, containing 0.15 mol/L NaCl, 0.05% Polysorbate 80, 0.03% Triton X-405, and 0.01% ProClin 150 (washing buffer) to remove unbound HTGL molecules. Then, anti-human HTGL monoclonal antibody, conjugated with horseradish peroxidase (HRP), was added to the microtiter plate wells, and the plate was incubated for 30 min at 25°C. The microtiter plate was washed three times with the washing buffer to remove unreacted HRP-labeled monoclonal antibody. A 100 μL aliquot of the color developing reagent solution was added to each well of the plate to assay the HRP activity, incubation was performed for 30 min at 25°C, and the reaction was terminated by addition of 100 μL of 1.8 N sulfuric acid to each well. The absorbance at 492 nm (with 620 nm as a reference wave length) was measured using an ELISA photometer. Pure human PHP-HTGL standards (20-μL samples equivalent to 0, 100, 200, 400, 600, and 800 ng/mL), diluted with heat inactivated human serum and 80 μL of the assay buffer, were placed in the microtiter plate wells coated with anti-human PHP-HTGL monoclonal antibody for the preparation of a standard curve; the subsequent steps were performed as described above. The concentration of the immunoreactive HTGL mass in each PHP sample was calculated by reference to the standard curve obtained from the six points of the human PHP-HTGL standards and expressed as nanogram of immunoreactive HTGL protein mass per milliliter of plasma.

Stability of Reagents Included in the HTGL ELISA Kit for Long-Term Storage

The HTGL ELISA kit consists of immobilized anti-human HTGL monoclonal antibody on microtiter wells, anti-human HTGL monoclonal antibody conjugated with HRP, assay buffer, enzyme substrate, washing buffer, and stop solution, as well as standard human PHP-HTGL. The shelf-life of the HTGL ELISA kit depends on the stability of these reagents, especially on those of immobilized antibody and HRP-labeled antibody prepared by the methods described in our previous paper.[13,14] In regard to standard HTGL, PHP including an immunological protein mass of 800 ng of HTGL per mL was subjected to lyophilization. The stability of the HTGL ELISA kit stored at 4°C for 2 years was monitored at intervals of 0, 3, 12, and 26 months, based on the method shown in Figure 1. The values of optical density of the standard HTGL calibration curve and the concentrations of HTGL immunoreactive mass in the control plasma samples stored in liquid nitrogen, measured by the HTGL ELISA, were used in the performance of ELISA as indicators for ascertaining periodic change.

RESULTS

Evaluation of HTGL ELISA MARUPI Kit

In the present study, the stability of HTGL immunoreactive mass in PHP in a long term of storage was ascertained using the immunological reactivity of HTGL as an indicator, which was monitored by the HTGL ELISA kit. Therefore, at first, the reliability of the HTGL ELISA kit was assured by precision and dilution tests. In Figure 2, a typical calibration curve of the HTGL ELISA kit is shown, ranging from 0 to 800 ng of HTGL mass concentration per mL of plasma. The lower detection limit of the ELISA was designated to be 10 ng/mL by a 2SD method. The precision study was carried out using two distinct samples containing different mass concentrations of human PHP-HTGL. As shown in Table 1, both intra- and inter-assay coefficients of variation of the immunoreactive HTGL mass concentrations for the two PHP samples were less than 2.2% and 5.1%, respectively. Dilution tests were carried out using three distinct PHP samples, ranging from 1,500 to 2,800 ng of human PHP-HTGL per mL of PHP. As shown in Figure 3, a linear dose-response curve was obtained over the range of 0–800 ng/mL for each of the samples. The results obtained in these tests indicate that the HTGL ELISA is an appropriate tool for measuring immunoreactive HTGL mass concentrations in PHP samples.

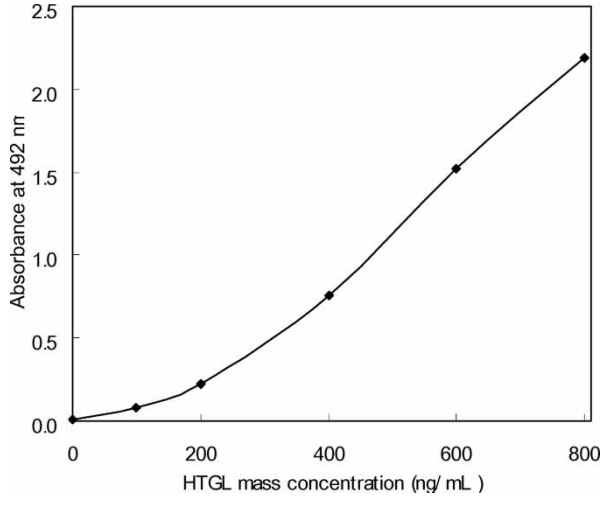

Figure 2. Calibration curve of human PHP-HTGL mass with the HTGL ELISA MARUPI kit. A purified human PHP-HTGL preparation was used as a standard material. Each plotted point represents the mean of three different measurements.

Table 1. Precision test for the quantification of immunoreactive HTGL mass concentration by the HTGL ELISA MARUPI kit. The HTGL mass in four PHP samples (A, B, C and D) was assayed by the HTGL ELISA MARUPI kit

	Sample	Assay number	Mean (SD) of HTGL mass (ng/mL)	Coefficient of variation (%)
Intra-assay	A	10	416.6 (9.1)	2.2
	B	10	352.2 (6.4)	1.8
Inter-assay	C	15	445.8 (14.9)	3.3
	D	15	401.3 (20.4)	5.1

Stability of Immunoreactive HTGL Protein Mass in the Liquid State

After the storage of human PHP in the liquid state, at 4°C and 25°C, the immunoreactive concentration of HTGL protein mass in PHP was measured with the HTGL ELISA MARUPI kit at 0, 2, 9, 24, 32, 48, and 72 hours after the start of storage. The results are shown in Figure 4. When stored at 4°C, the immunological activity decreased by approximately 5% in 24 hours and by approximately 10% at 72 hours. On the other hand, in the case of storage at 25°C, the immunological activity decreased by approximately 20% in 24 hours and approximately 40% at 48 hours, when it is compared with the original immunological acitivity.

Stability of Immunoreactive HTGL Protein Mass in the Frozen State

After the storage of human PHP in the frozen state at −20, −80°C, or in liquid nitrogen, the immunological activity of HTGL protein mass in PHP was measured with the ELISA at 1, 2, 3, and 4 months after the start of storage. The results are shown in Figure 5. No decrease in immunological activity of HTGL protein mass was observed when the PHP was stored at −80°C for 2 months or in liquid nitrogen for 6 months. On the other hand, when it was stored at −20°C, an approximate 10% decrease in immunological activity of HTGL was noted during one month of storage. In frozen state, storage at lower temperature, such as in liquid nitrogen, makes it possible to keep the original immunoreactivity of HTGL protein for a long period.

Stability of Immunoreactive HTGL Protein Mass in the Lyophilized State

In Figure 6, the results of stability of immunoreactive HTGL protein mass in PHP in the lyophilized state, after storage at −80, −20, 4, 25, and 37°C are

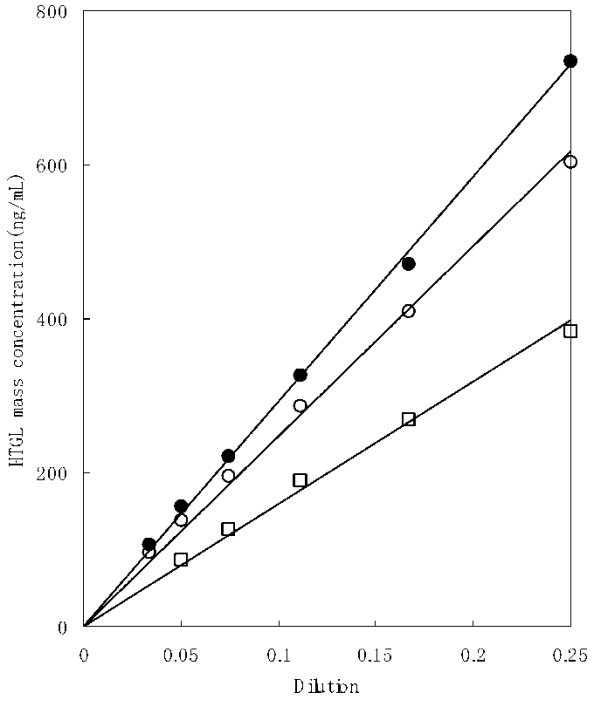

Figure 3. Dose-response curves of HTGL mass measured by the HTGL ELISA MARUPI kit. Three PHP samples containing immunoreactive HTGL mass concentrations of 734 ng/mL (●), 604 ng/mL (○), and 384 ng/mL (□) were diluted with control human serum as indicated. Each plotted point represents the mean of duplicates, and the duplicate values differed by less than 5%.

shown. When stored at −80, −20 and 4°C, no remarkable decrease in immunological activity of HTGL protein was observed after 26 months of storage. On the other hand, in the case of storage at 25°C, the decrease of immunological activity of HTGL was observed to be approximately 70% in 18 months. In the case of storage at 37°C, a decrease by 50% was attained in 4 months. The lyophilized PHP preparation stored at 37°C could not be completely reconstituted after 5 months or longer storage, making it impossible to quantify HTGL immunoreactive mass concentration.

Stability of Reagents in the HTGL ELISA Kit during Long-Term Storage

The stability of HTGL ELISA, consisting of reagents such as immobilized anti-HTGL monoclonal antibody, HRP-labeled anti-HTGL monoclonal antibody, substrate, stop solution, and washing solution in a storage

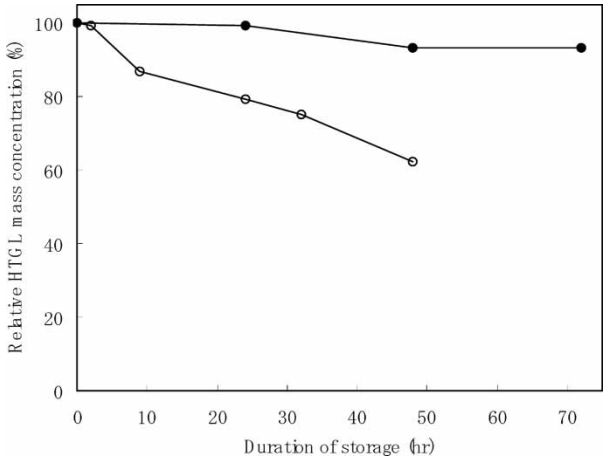

Figure 4. Periodical change of the immunoreactive HTGL mass concentration in human PHP stored in a liquid state at 4°C (●) or 25°C (○). After storage for the indicated time, the immunoreactive HTGL mass concentration was measured by the HTGL ELISA MARUPI kit.

condition at 4°C for over 2 years was ascertained by monitoring the periodic changes of optical density of the standard curve and the measured values of immunoreactive HTGL mass concentration in the control plasma samples stored frozen in liquid nitrogen, which condition is thought to be the best for the purpose of keeping HTGL immunoreactivity stable for a long period. As shown in Figure 7, the optical density at each concentration of

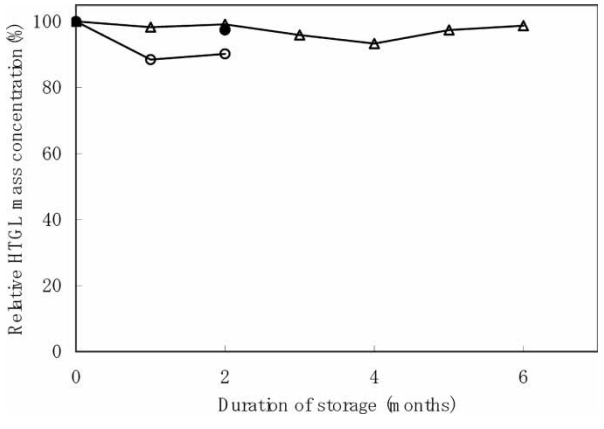

Figure 5. Periodic change of the immunoreactive HTGL mass concentration in human PHP stored in a frozen state at −20°C (○), −80°C (●) or in liquid nitrogen (△). After storage for the indicated duration, the immunoreactive HTGL mass concentration was measured by the HTGL ELISA MARUPI kit.

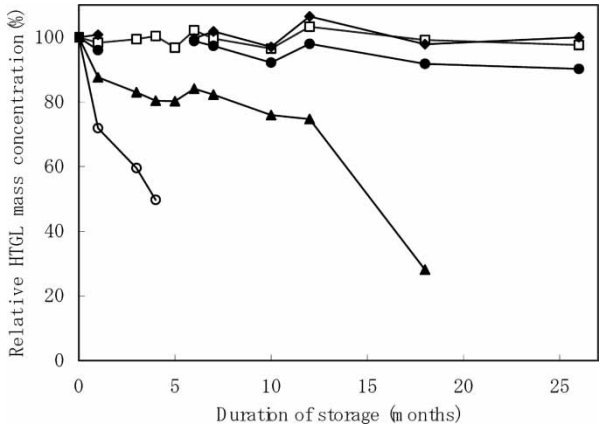

Figure 6. Periodic change of the immunoreactive HTGL mass concentration in human PHP stored in a lyophilized state at −80°C (□), −20°C (◆), 4°C (●), 25°C (▲) or 37°C (○). After storage for the indicated duration, the immunoreactive HTGL mass concentration was measured by the HTGL ELISA MARUPI kit.

standard HTGL in the respective standard curves during this stability test seems to be approximately 50% decreased; however, the concentrations of immunological HTGL mass was almost constant for 26 months (shown in Figure 8).

DISCUSSION

In the present work, we studied changes in the immunoreactive HTGL mass during storage under various conditions. In the liquid state, immunological

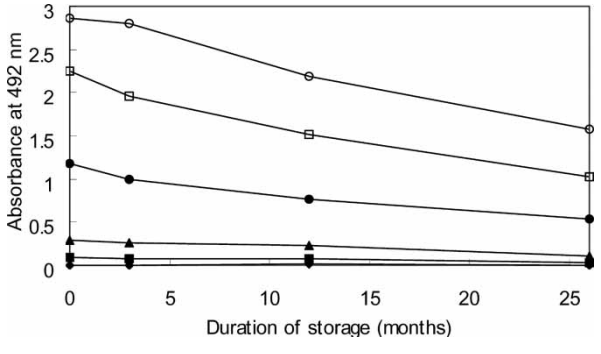

Figure 7. Periodic change of the optical density at each concentration of standard HTGL in the respective standard curves in the duration of storage. Standard HTGL: 0 ng/mL (◆), 100 ng/mL (■), 200 ng/mL (▲), 400 ng/mL (●), 600 ng/mL (□) and 800 ng/mL (○).

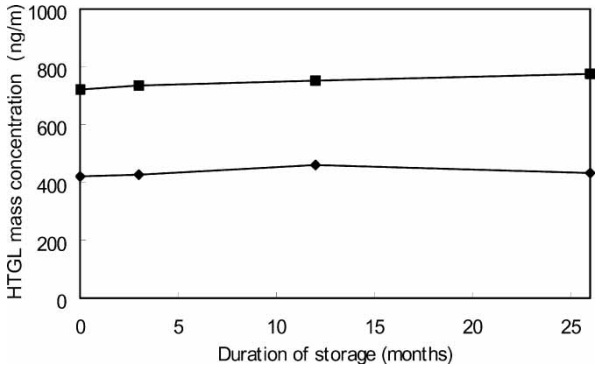

Figure 8. Periodic change of the immunoreactive HTGL mass concentration in PHP sample in the duration of storage. Two PHP samples containing immunoreactive HTGL mass concentrations of 722 ng/mL (■) and 421 ng/mL (◆) were stored in liquid nitrogen.

activity of the enzyme decreased by approximately 40% at 48 hours in the case of storage at 25°C. In a previous reference, it was reported that the enzymatic activity of HTGL in 10 mmol/L phosphate buffer was lost almost completely within 24 hours after the start of storage at 20°C.[15] They showed an effectiveness of a detergent Triton N-101 for stabilizing the enzyme activity of HTGL; however, a periodic decrease of the catalytical activity was observed within 24 hours. In comparison with the enzymatic activity, the immunological activity of HTGL protein mass measured by the HTGL ELISA seems to be relatively stable. This is probably because the enzymatic activity of HTGL can be affected even by slight denaturation of the enzyme molecule, while the immunological activity of HTGL is well-preserved as the epitopes present in HTGL molecule remain to be reactive towards the antibodies.

In the previous reports, lyophilization was successfully used for a long-term storage of physiologically active and unstable proteins, such as creatine kinase isoenzymes,[16] elastase,[17] phosphofructokinase,[18] α-amylase,[19] interleukin-11,[20] etc. In addition to lyophilization, it is well known that sugars are effective for stabilizing proteins. Carpenter[18] and Garzon-Rodriguez[20] used sugars as stabilizers in their investigation. As HTGL is one of the members of heparin-binding proteins, HTGL may be stabilized by heparin, a mucopolysaccharide derived from porcine enteric mucosa, administered intravenously when PHP was collected. In the present study, we, therefore, tested lyophilization of PHP as a means of storing HTGL protein to keep the immunological activity longer. The lyophilized PHP containing HTGL protein retained immunological activity equivalent to the original level for at least two years when stored at temperatures below 4°C. We, thus, confirmed that lyophilization allows a long-term storage of PHP containing HTGL immunoreactive protein mass. Using this

technique, it is now possible to supply large amounts of lyophilized PHP containing HTGL protein used as a stable standard material. Therefore, it would be possible to verify the comparability of results from different laboratories for inter-laboratory quality control. Then, lyophilized PHP has been utilized as a standard material for HTGL immunoassay with the HTGL ELISA MARUPI kit.

We ascertained the long-term stability of the HTGL ELISA kit, which includes lyophilized PHP-HTGL, an immobilized anti-human HTGL monoclonal antibody, and HRP-labeled anti-human HTGL monoclonal antibody. The optical density at each concentration of standard HTGL in the respective standard curves during this stability test seems to be approximately 50% decreased; however, the concentrations of immunoreactive HTGL protein mass were almost constant for 26 months. Decline of the optical density at each concentration of standard HTGL, kept in long-term storage, was thought to be dependent upon loss of the enzymatic activity of HRP conjugated with anti-HTGL monoclonal antibody, but not of immunological reactivity of HTGL in lyophilized preparation, because the immunoreactive HTGL protein mass concentration in the control plasma samples stored in liquid nitrogen measured by each standard curve was constant. Achievement of a long shelf-life of more than 2 years for reagents included in the HTGL ELISA kit enables us to carry out measurement of immunoreactive HTGL protein mass concentration in both pre-heparin plasma and PHP samples with the HTGL ELISA MARUPI kit, practically, and it also makes it possible for us to manufacture the HTGL ELISA kit with a long shelf-life on an industrial scale. To our knowledge, this is the first case to succeed in keeping the immunological activity of HTGL protein stable in long-term storage of more than 2 years.

It is concluded that HTGL in PHP was stabilized by lyophilization and can be used as the standard material for HTGL ELISA, and that the HTGL ELISA kit has a long shelf-life of more than two years.

ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; HTGL, hepatic triglyceride lipase; IDL, intermediate density lipoprotein; LPL, lipoprotein lipase; PHP, postheparin plasma; TG, triglyceride; VLDL, very low density lipoprotein.

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