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ORIGINAL RESEARCH

Determination of lipoprotein lipase activity in post heparin plasma of streptozotocin-induced diabetic rats by high-performance liquid chromatography with fluorescence detection

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ABSTRACT: The activity of lipoprotein lipase (LPL), an enzyme responsible for lipoprotein metabolism, would vary in diseases and metabolic disorders. For determination of LPL activity, a highly sensitive high performance liquid chromatography (HPLC) method using a fluorescent reagent, 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) was applied to determinate the oleic acid (OA) generated from triolein by LPL activity without multiple solvents extraction step. We studied the optimal conditons of the reaction including the effect of emulsifiers, deproteinizing solvents, and the concentration of bovine serum albumin (BSA). Ten millimolar concentrations of triolein, 5% of BSA, 1% of Gum arabic (GA), and acetonitrile showed the optimum conditions for measuring the LPL activity. The accuracy values for the determination of LPL activity in 10 μL of rat post heparin plasma were 108.73 ~ 114.36%, and the intra- and inter-day precision values were within 1.28% and 2.91%, respectively. The limit of detection was about 4.53 nM (signal-to-noise ratio 3).

The proposed method was applied to determination of LPL activity in post heparin plasma of normal and streptozotocin-induced diabetic rats associated with 52.3% reduction. The established assay system could be used for determining LPL activity in different physiological and pathological conditions to clarify the relationship between LPL activity and diabetes mellitus.

KEYWORDS: lipoprotein lipase; fluorescence; HPLC; NBD-PZ; emulsifiers; BSA; STZ-induced diabetic rats

INTRODUCTION

Lipoprotein lipase (LPL, EC 3.1.1.34), a 448-residue glycoprotein, is responsible for the metabolism of triglyceride-rich lipoproteins in plasma. It is synthesized in tissue parenchymal cells and is secreted and transported to the capillary endothelium, where it binds to heparin sulfate (Goldberg, 1996; Santamarina-Fojo and Dugi, 1994). Abundant amounts of LPL are found mainly in organs with a high demand for free fatty acids (FFAs) such as adipose tissues, heart and skeletal muscles (Semenkovich *et al.*, 1989; Zechner, 1997). Studies have indicated that important roles of this rate-limiting enzyme exist in lipoprotein metabolism and clinical disorders, including diabetes mellitus, cardiovascular diseases, diet-related metabolic disorders and lipoprotein metabolism-related disorders such as hyperlipoproteinemia, and familial hypercholesterolemia (Eckel, 1989; Mahley *et al.*, 1991; Scheen, 1999). These observations not only provide insights into the importance of lipoprotein lipase, but also implicate the potential role of LPL activity in the pathogenesis of dyslipidemia in the condition associated with insulin resistance (Jeppesen *et al.*, 1995; Nikkilä *et al.*, 1977; Pulawa and Eckel, 2002; Pykalisto *et al.*, 1975; Taskinen *et al.*, 1982). A number of studies have shown various results that have been difficult to verify due to methodological differences such as the type of substrates measured, variable reaction medium and conditions, and different sample characteristics (Maheux *et al.*, 1997; Sartippour and Renier, 2000; Yamazaki *et al.*, 2002). Over the last

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Abbreviations used: BSA, bovine serum albumin; BuOH, *n*-butanol; DPDS, 2,2′-dipyridyl disulfide; EtOH, ethanol; FFAs, free fatty acids; GA, gum arabic; HPLC, high performance liquid chromatography; LPL, lipoprotein lipase; MeCN, acetonitrile; MeOH, methanol; NBD-PZ, 4-nitro-7-piperazino-2,1,3-benzoxadiazole; OA, oleic acid; STZ, streptozotocin; TPP, triphenylphosphine; TFA, trifluoroacetic acid.

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decade, the standard analytical system of LPL is still limited. In addition, recent studies have failed to examine the effect of the composition of reaction medium on the enzyme reaction, since changing the contents of the emulsion could theoretically produce results. However, this may cause methodological errors.

To accurately determine the LPL activity, several factors have been considered in developing appropriate analytical assays. These factors include choosing the substrate of LPL to be as physiologically specific as possible and the chemicals representing the end products to have enough particle surfaces for interfacial activation and co-enzymes, and the assay to be as sensitive as possible (Bauer, 1988; Gupta *et al.*, 2003; Wang *et al.*, 1982). To date, numerous methods, including titrimetric (Hoppe and Theimer, 1996), radiometric (Nilsson-Ehle and Schotz, 1976), fluorimetric (Duque *et al.*, 1996) and HPLC assays (Eguchi, 2002), have been developed. Among these methods, the radioisotope-labeled substrate for LPL has been widely used to evaluate LPL activity in various physiological and pathological conditions. However, there are some drawbacks associated with this method, such as its inability to completely separate FFAs and radioactive substrates, which may affect the precision and accuracy of analysis (Belfrage and Vaughan, 1969). Other difficulties, including exposed to radiation hazards from radioactive materials, disposal of radioactive isotopes, restriction to licensed laboratories and the short half-life of the labeled reagents, have also been mentioned (Ball *et al.*, 2002). Many commercial kits with fluorescent-labeled substrates may solve the hazard from radioisotopes but they exhibit time-limit reactivity and high background disturbances and are not suitable for long-term study (Beisson *et al.*, 2000; Sartippour and Renier, 2000). A better analysis for LPL activity using HPLC has been developed by Eguchi (2002). However, adequate concentrations of emulsifier or BSA, which factor is dominant in LPL reactions, have not been investigated.

Considering these problems, an assay system which is able to detect and quantify fatty acid generated by LPL using HPLC plus fluorimetric detection, without using the step of sample extraction and producing unstable synthesized substrates, has been established. The present study was carried out to investigate the importance of LPL reaction medium. More specifically, the goal of this study was to evaluate the influence of emulsifiers, serum albumin, and solvent system on the proposed assay for plasma LPL activity. For this purpose, LPL activity was measured in reaction media and conditions. Subsequently, the proposed method was applied to determine the activity of LPL in normal and streptozotocin (STZ)-induced diabetic rats to establish an appropriate model for elucidating the character of LPL activity in plasma homeostasis of diabetes mellitus.

EXPERIMENTAL

Chemicals. LPL from bovine milk (0.94 mg protein/mL), GA, sodium deoxycholate, Tween® 20, Tween® 40, Tween® 80, BSA, heparin, streptozotocin (STZ), triolein, OA ($C_{18:1}$), and heptadecanoic acid (margaric acid, $C_{17:0}$) were purchased from Sigma Chemical (St Louis, MO, USA). Tris–HCl buffer was obtained from Calbiochem, which belongs to EMD Biosciences (Darmstadt, Germany). Sodium chloride, sodium hydroxide and citric acid monohydrate were purchased from Nacalai Tesque (Kyoto, Japan). 4-Nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), triphenylphosphine (TPP) and 2,2′-dipyridyl disulfide (DPDS) were obtained from Tokyo Kasei Chemicals (Tokyo, Japan). Trifluoroacetic acid (TFA) was purchased from Riedel-de Haën (Seelze, Germany). HPLC-grade acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH) and *n*-butanol (BuOH) were from Merck (Darmstadt, Germany).

Preparation of enzyme source from normal and STZ-induced diabetic rats. Animal experiments were approved by the Laboratory Animal Research Committee of Taipei Medical University. Wistar male rats (Laboratory Animal Center of National Taiwan University, Taipei, Taiwan) weighing 200–250 g were used in the experiment and kept in an environmentally controlled room with food and tap water *ad libitum*. To induce diabetic rats, streptozotocin dissolved in 50 mM citrate buffer (pH = 4.5) was administered intraperitoneally at a dose of 100 mg/kg after fasting for 12 h as described in previous study (Lee *et al.*, 2005). Diabetes mellitus was confirmed by documenting persistent hyperglycemia that was measured using a commercial kit (Lifescan, CA, USA). Lipoprotein lipases that bound to heparin sulfate on the endothelial wall were released by injecting 100 IU/kg of heparin intravenously. After 10 min, the blood of experimental animals from normal and diabetic rats was drawn from the tail vein and centrifuged with Mikro 22R Centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 10,000 rpm for 10 min at 4°C immediately. The supernatant was used as an enzyme source and stored at –20°C.

Proceeding of the enzyme reaction. The assay of LPL activity is based on fluorimetric measurement of derivatized fatty acids formed enzymatically from the external added substrate, triolein (Eguchi, 2002). Experiments for the selection of substrate concentrations, emulsifiers, BSA concentrations and deproteinized solvents were conducted for optimizing the assay system. First of all, five types of emulsifiers were used for emulsifying the triolein substrate, including GA, sodium deoxycholate, Tween® 20, 40 and 80. Weighted emulsifiers were mixed and sonicated with the buffer containing 0.2 M Tris–HCl, and 0.1 M NaCl (pH adjusted to 8.2 with 1 M NaOH) until homogenization. The concentration of each emulsifier was set at 1 and 5% (w/v). Among the chosen emulsifiers, GA was commonly used for stabilized triolein emulsion. Consequently, GA concentrations of 0, 0.25, 0.5, 1, 2, 4, 6 and 8% were also evaluated. Pure triolein was then emulsified with the buffer made with different types and concentrations of emulsifiers according to the descriptions above. Moreover, the proportion of BSA in the reaction buffer was investigated at six concentrations (0, 1.25, 2.5, 5, 7.5 and

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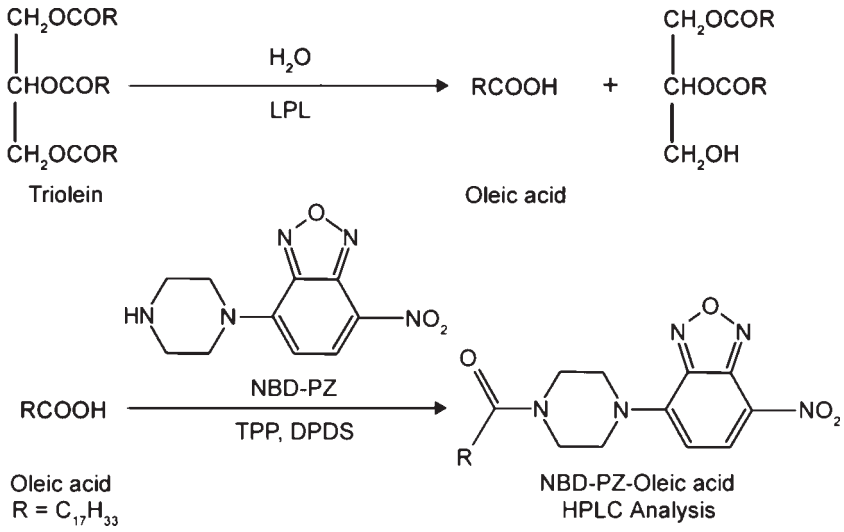

Figure 1. The principle for determination of LPL activity. OA, the product generated after triolein hydrolyzed by LPL, was derivatized with NBD-PZ and quantified by a HPLC system.

10%). Ten micro-liters of enzyme source was combined with triolein emulsion to a total volume of 100 μL and incubated for 30 min at 37°C. The enzyme reaction was terminated and deproteinized by addition of 900 μL of acetonitrile and centrifuged directly at 3300 rpm for 5 min at 25°C. Equal volumes of MeOH, EtOH, and BuOH were tested as well. The supernatant in each experiment was continually derivatized and analyzed as follows.

Derivatization of oleic acid with NBD-PZ. OA released from triolein by LPL activity was derivatized with a fluorescence reagent, NBD-PZ, via the principle of the derivative reaction, as shown in Fig. 1. The procedures were carried out according to our previous method with minor modifications (Tsai *et al.*, 2003). Briefly, 100 μL of 2 mM NBD-PZ in MeCN mixed with 10 μL each of 280 mM TPP and DPDS in MeCN was supplemented with the resulting reaction. Ten microliters of 0.5 mM margaric acid dissolved in MeCN was added as an internal standard (IS) (Sparreboom *et al.*, 1996). The derivatization reaction was incubated for 20 min at 40°C and then terminated by the addition of 450 μL of 0.1% TFA in distilled deionized water. An Empore SDB-RPS cartridge (3M, St Paul, MN, USA) was used to remove the excess derivative reagents. The cartridge was preconditioned with 100 μL of MeCN and loaded with 100 μL of the resulting solution. One-hundred microliters of MeCN were loaded subsequently for thorough elution. Two portions of the eluent were combined for HPLC analysis. All procedures were performed using disposable polypropylene brown Eppendorf tubes (1.5 mL) to speed the experiments.

HPLC conditions. The samples were subjected to a reversed-phase HPLC system consisted of an intelligent auto-sampler (AS-950; Jasco Co., Tokyo, Japan) equipped with a 20 μL sample loop and a pump (L-7100; Hitachi, Tokyo, Japan). The composition of the mobile phase was acetonitrile–methanol–water (50:35:15, v/v/v) performed for 30 min at a flow rate of 0.7 mL/min. NBD-PZ labeled fatty acids were separated on an C_8 column (YMC-Pack Pro C_8, 150 × 4.6 mm i.d., spherical 5 μm, 120 Å, YMC Co., Kyoto, Japan) and detected with a fluorescence detector (F-1000; Hitachi, Tokyo, Japan) at 491 and 547 nm excitation and emission wavelengths, respectively. The results were analyzed using a D-2500 chromato-integrator (Hitachi, Tokyo, Japan).

Validation study. For quantitation of the OA formation by the enzyme action, a calibration curve was constructed. One-hundred microliters of standard OA at concentrations of 0, 0.50, 1.00, 2.00, 4.00, 6.00 and 8.00 mM was brought up to 1000 μL with MeCN and the same derivatization procedures (n = 5) were subsequently carried out. The calibration curve was plotted from the peak area ratio of derivative of OA to a derivative of margaric acid against the concentration of OA dissolved in MeCN. Spiked amounts of OA (0, 1, 2 and 3 mM) into the reaction mixture were performed to estimate the precision and accuracy of the present analytical method. The intra- and inter-day precision expressed as the relative standard derivation (RSD) was assessed by analyzing six samples for one day and on six different days. The accuracy of this method was presented as the calculated recovery.

RESULTS AND DISCUSSION

Determination of LPL activity by the novel HPLC analysis

The existing assays for determination of lipoprotein lipase activity have been developed over three decades (Baginsky and Brown, 1977, 1979; Nilsson-Ehle and Schotz, 1976). Recently, some single nucleotide polymorphisms were discovered from human LPL gene (Hoh and Hodge, 2000; Nickerson *et al.*, 1998; Templeton *et al.*, 2000). Analysis for enzyme activity of mutated LPL gene needs more precise and accurate

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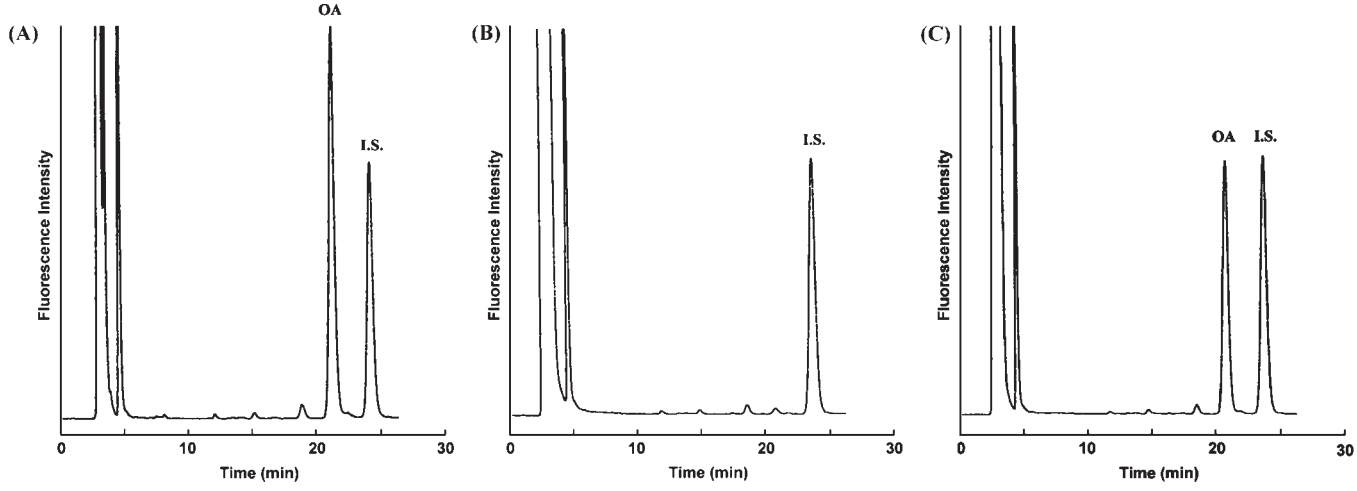

Figure 2. HPLC analysis of NBD-PZ labeled OA which was liberated by commercial LPL activity. (A) Chromatogram of standard OA. (B) Chromatogram of triolein only. (C) Chromatogram of triolein with the presence of commercial LPL.

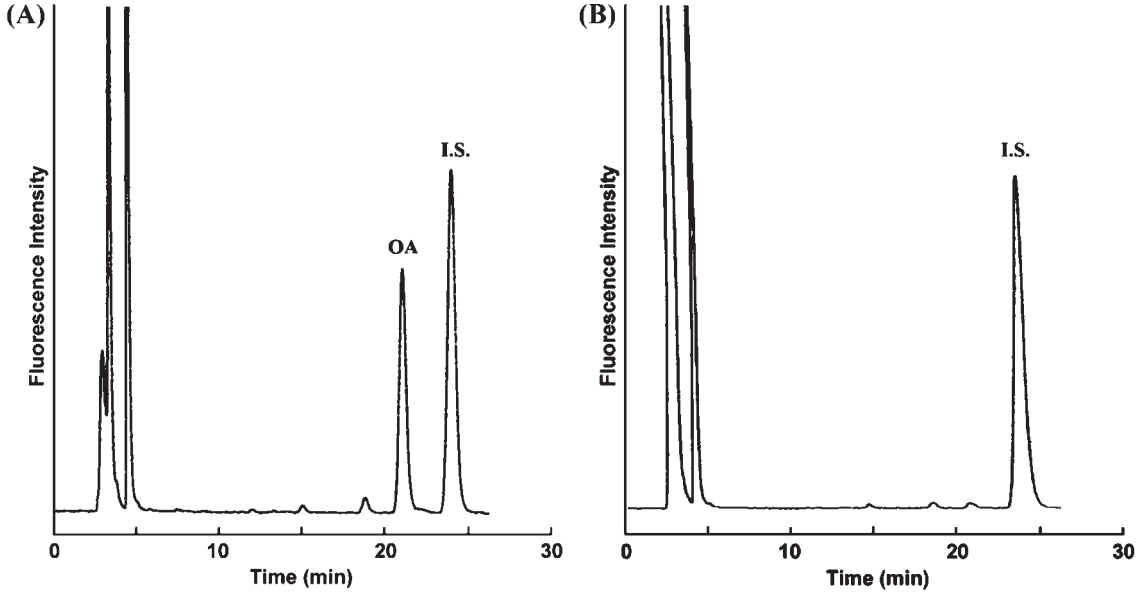

Figure 3. HPLC analysis of NBD-PZ labeled OA which was liberated by LPL activity in rat post-heparin plasma. (A) The peak representing the OA derivative on chromatogram could be found with the presence of LPL. (B) The peak disappeared with MeCN pretreatment since LPL was deproteinized by MeCN.

methods with good sensitivity. As pointed out in other HPLC-based enzymatic assays, the direct analysis of enzyme action, once separated from substrate and other interfering substances, offers clear results (Eguchi, 2002). The NBD–oleic acid reaction was fast and started immediately on vortex mixing (data not shown). Figure 2 shows the chromatograms of the standard NBD–OA derivative, triolein only as a blank and NBD-PZ-labeled OA derived from sample. The NBD–OA and NBD–margaric acid derivatives were eluted at approximately 22 and 24 min, respectively. Under addition of 4 mM OA as standard control, it produced a significant amount of NBD–OA in 22 min [Fig. 2(A)], whereas the blank with 10 μL double-distilled deionized water instead of post heparin plasma exhibited no background interference [Fig. 2(B)]. As shown in Fig. 2(C), the derivatized OA by commercial bovine milk LPL was detected and completely separated from other impurities using the reversed-phase HPLC system described in the Experimental section. Figure 3(A) indicated that the successful detection of OA was produced by LPL activity in post-heparin plasma of normal rats. NBD–OA was not found when LPL was pre-denatured in post-heparin plasma of normal rats [Fig. 3(B)]. Enzyme activity was calculated according to the OA formation and expressed as milliunits in

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Table 1. The emulsification suitable for LPL by different emulsifiers were evaluated by judging the formation of OA ($n = 5$)

Final concentration (w/v %)	Oleic acid formation (% of control)	
	1%	5%
None	100	
Gum arabic	197.6 ± 2.0	113.6 ± 3.8
Sodium deoxycholate	82.8 ± 1.0	81.8 ± 0.0
Tween 20	96.3 ± 1.4	97.3 ± 1.5
Tween 40	95.0 ± 0.5	86.0 ± 1.8
Tween 80	279.9 ± 2.1	409.6 ± 3.2

the sample assayed. One milliunit of enzyme activity is defined as the release of 1 nmol of OA per microliter of plasma per minute at 37°C. The enzymatic reaction was linear for at least 2 h at 37°C (data not shown).

Selection of emulsifiers

Numerous experimental and clinical studies have been conducted to evaluate the role of LPL activity in relation to hyperlipoproteinemia, atherosclerosis and metabolic disorders such as obesity and diabetes. Since there are various assay systems and inconsistent components of substrate emulsion, it was difficult to evaluate and compare results for those researches (Maheux *et al.*, 1997; Sartippour and Renier, 2000; Yamazaki *et al.*, 2002). For improvement and optimization of the assay system, the effects of emulsifiers, BSA and deproteinized solvents on sample were investigated. As shown in Table 1, using Tween® 80 as emulsifier yielded the greatest enzyme activity at both concentrations (1 and 5%). Tween® 80 (polyoxyethylene sorbitan monooleate) consists of monooleate and has been reported to be a substrate for lipase (Surinenaite *et al.*, 2002); it may contribute to the OA determination but interferes with the major hydrolysis of triolein. Unlike Tween® 80, neither Tween® 20 nor 40 was suitable for emulsification, although they did not cause interference (Archibald, 1946). LPL activity was not detected when emulsifying with the anionic detergent, sodium deoxycholate (Table 2). This phenomenon was also observed in the study of Yadav, who demonstrated that anionic detergents that might repel the lipase from the interface or bind to the enzyme or even encapsulate the enzyme so that the substrate-binding site is blocked (Yadav *et al.*, 1998). With 10 mM triolein as the substrate, GA (1%) seemed to be the best emulsifier for determination of LPL activity.

1% Gum arabic provides the most efficient emulsion of the substrate

Since triolein dispersed in 1% GA instead of 5% results in the increase of enzyme activity, the optimization of GA concentrations for LPL activity in the assay emulsion was further investigated. As shown in Fig. 4, LPL activity showed a rising tendency until GA concentration reached 1%, then slowly decreased and remained stable from 2 to 8%. Using the same substrate concentration, identical conditions for emulsification of triolein and the same enzyme preparation, the LPL activity was clearly affected by concentration of emulsifier. Apparently, the enhanced emulsifying capacity of increased

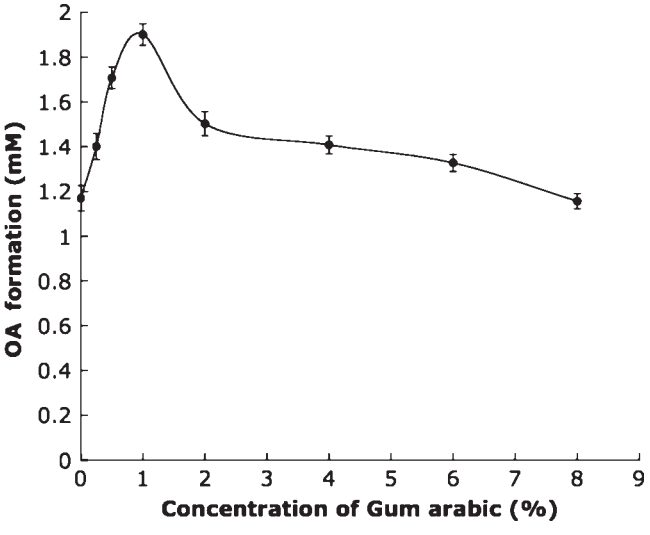

Figure 4. Influence of concentration of gum arabic. Maximum activity of OA was observed in emulsification with 1% of gum arabic. Overloaded emulsifier caused a decrease in release of OA ($n = 5$).

Table 2. Precision and accuracy of oleic acid released by LPL activity ($n = 6$)

		Oleic acid added (mM)			
		0.00	1.00	2.00	3.00
Intraday	Measured (mM)	2.18	3.31	4.36	5.44
	CV (%)	1.28%	1.20%	0.97%	0.90%
	Recovery (%)	—	113.48%	108.75%	108.73%
Interday	Measured (mM)	2.19	3.33	4.36	5.54
	CV (%)	2.91%	0.58%	0.50%	0.39%
	Recovery (%)	—	114.36%	108.77%	111.61%

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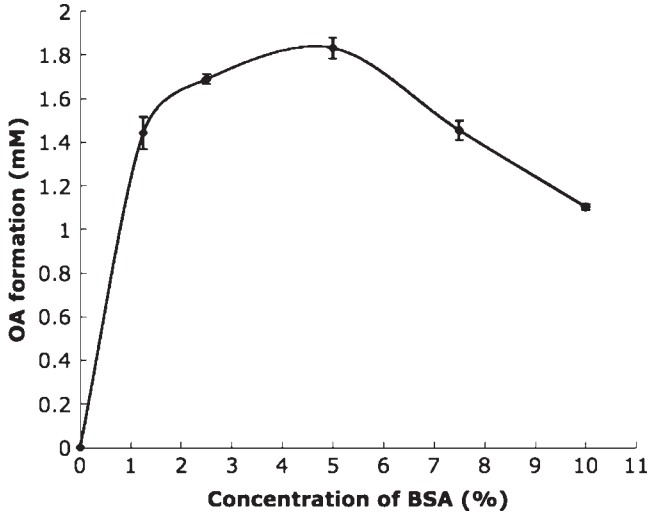

Figure 5. Influence of concentration of BSA. Maximum activity of OA was observed in emulsification with the presence of 5% of BSA. BSA is a cofactor in LPL reaction but overloaded BSA might interfere adsorption of enzyme ($n = 5$).

GA concentrations increased the surface area of the oil–water interface, and, therefore, LPL activity is at optimum. It was surprising that continuously increased GA concentrations did not increase the enzyme activity as expected. In contrast, overloaded GA caused a decrease in activity of LPL. Possibly, OA produced by LPL when GA was overloaded in high concentrations may retain in emulsified triolein particles. After adding deproteinized solvent to terminate the LPL reaction, some OA was also removed with the emulsified triolein particle by centrifugation (Hoppe and Theimer, 1996).

5% BSA is critical for effective determination of LPL activity

The dependence of enzyme activity for BSA was also studied. LPL activity was undetectable with triolein emulsions stabilized only by the emulsifying agent, GA, in the absence of BSA in the test solution (Fig. 5). The greatest catalytic activity was observed while 5% of BSA existed in the reaction buffer. However, excessive addition of BSA (up to 10%) caused a decrease in LPL activity. Traditionally, serum albumin has been shown to interact with the lipase-emulsified substrate reaction in two different ways. It protects the lipase from irreversible inactivation at low concentration. On the other hand, it inhibits enzyme activity by blocking the substrate surface (Tsujita *et al.*, 1996). Hence, BSA prevents the accumulation of the released OA at the water–oil interface of the emulsion which has been reported to inhibit enzyme action (Blanchette-Mackie and Scow, 1976; Karpe *et al.*, 1992). Thus, the concentration of serum albumin was important for determination of LPL activity. It was reported that activators such as apolipoprotein CII was necessary for LPL reaction (LaRosa *et al.*, 1970). To provide the co-factor, apolipoprotein CII, serum obtained from animal or human was heated to eliminate endogenous lipolytic activity (Nilsson-Ehle and Schotz, 1976) or directly purified apolipoprotein CII from serum by complex procedures (Lobo and Wilton, 1997). This heat-inactivated serum was added to the reaction emulsion in our experiment; however, the effect of apolipoprotein CII on LPL activity was limited (data not shown). Lobo and Wilton (1997) indicated that LPL activity in rat serum was still detected without heat-inactivated serum *in vitro*. To simplify the assay system, heat-inactivated serum was not used in this study. In addition, we proposed that the BSA might provide the synergic effect which the apolipoprotein CII had on enzyme action. Therefore, the presence of BSA was a key factor for determination of LPL activity.

Solvent effects

Deproteinized solvents were tested as another important parameter in establishing the assay system of LPL activity. Figure 6 showed that the value of OA liberated from the enzyme reaction was lower in alcoholic solvents than in MeCN ($p < 0.001$). It may result from the esterification of OA and alcoholic solvent molecules activated by the plasma lipases (Sharma *et al.*, 2001). The usage of NBD-PZ dissolved in MeCN as the fluorescent agent also gave a better reactivity

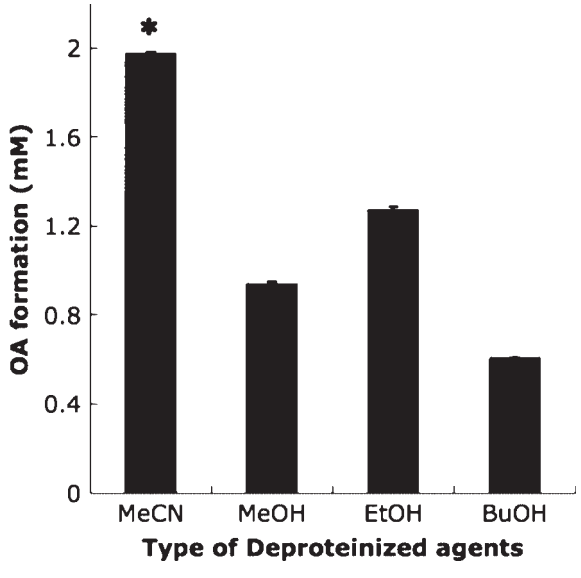

Figure 6. Optimization of the deproteinized agents. Four types of solvents including MeOH, EtOH, BuOH and MeCN were used. MeCN was found to dissolve the most amount of OA released by LPL compared with other three solvents. Analysis was performed by a two-tailed independent student's *t*-test: * $p < 0.001$ compared with other types ($n = 6$).

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(Schneider *et al.*, 1991) and stability compared with other derivative agents or solvents (Eguchi, 2002). The fatty acid produced during the incubations was conventionally isolated using a modification of liquid–liquid partition system described by Belfrage and Vaughan (1969). The reactions were stopped by addition of methanol-chloroform-heptane 1.41/1.25/1 (v/v/v) followed by adding potassium carbonate-borate buffer (pH = 10.5) (Belfrage and Vaughan, 1969). The extraction and all subsequent procedures were complicated, whereas the deproteinized solvent used in our assay was not only employed easily and eliminated contamination but also gave good recovery results. After optimization of the assay system, three components of the assays should be considered: the selection for type and concentration of emulsifiers, the proportion of BSA and the deproteinization of extraction solvents. Three of them cannot be left out of discussion because the complex mechanism of lipolysis at the interface is between the emulsified substrate and the water-soluble enzyme.

Validation study

The established fluorescent HPLC assay system for the measurement of OA was sensitive and highly reproducible, associated with an efficient chromatographic separation of OA from triolein and other similar fatty acids. The calibration curve for OA showed a good linearity within 8 mM. Slope and intercept, the standard error mean of the slope and intercept, and the coefficients of determination were as follows: $y = (0.2909 \pm 0.0046)x - (0.0085 \pm 0.0069)$, $r^2 = 0.9997$. The slope and the *y*-intercept values introduced in the equation represent the mean of five different curves on the five different days. The limit of detection was found to be 4.53 nM for OA in our analytical system. The precision and accuracy of data for OA spiked into the reaction mixture are shown in Table 2. The recoveries (mean ± SEM, $n = 6$) at three different concentrations were 108.73–114.36%. The intra- and inter-day precision values expressed as RSD were smaller than 3%. The statistical validation indicates that the assay is satisfactory for the practical use.

Determination of LPL activity in post-heparin plasma of diabetic rats

Eguchi (2002) reported an HPLC method, in which the LPL activity was detected by reacting 9-anthryldiazomethane (ADAM) with enzyme-generated OA, successfully determining the LPL activity in human plasma. However, the half-life of ADAM solution is only one week at –20°C, and an extraction step is necessary for the sample preparation (Eguchi, 2002). The short half-life may have some influences on accuracy and precision. In our proposed method, NBD-PZ is used as a derivative reagent, which is more stable than ADAM. Furthermore, the NBD-PZ derivative was monitored at excitation 491 nm and emission 547 nm. Both of them are long wavelengths which could avoid the interferences caused by autofluorescence in biological samples. The conditions of enzyme reaction were also optimized in this study. Eguchi's method indicated that 4.5% of GA inhibited the enzyme activity, whereas 1% of GA emulsifier expressed the greatest efficacy.

Finally, the proposed HPLC method was applied to determine LPL activity in the post-heparin plasma of normal and diabetic rats. Because much lower amounts or activity of total LPL were found in the diabetic stage than in the normal situation (Kovar *et al.*, 2004), even in the transgenic mice with mutant LPL (Hoh and Hodge, 2000; Nickerson *et al.*, 1998; Templeton *et al.*, 2000). Thus, a highly sensitive method for the determination of circulation LPL activity should be useful. OA formation by LPL activity in normal and diabetic rat plasma was derivatized with NBD-PZ, separated on the C_8 column, and determined fluorimetrically. Using our assay system, 10 μL of plasma volume was sufficient for LPL activity determination. After receiving STZ for 1 week, significant hyperglycemia determined by the meter and strips was observed in the diabetic rats (494.7 ± 5.3 vs 115.0 ± 6.2 mg/dL in the normal group, $p < 0.001$). Figure 7 showed that the LPL activity of STZ-induced diabetic rats was markedly decreased compared with the normal rats (0.236 ± 0.041 vs 0.451 ± 0.041 mU in the normal group, $p < 0.01$). These

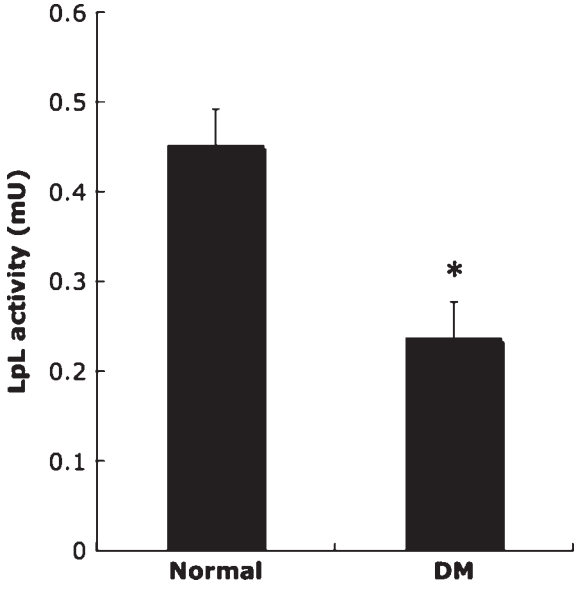

Figure 7. The LPL activity of normal and STZ-induced diabetic rats. LPL activity of STZ-induced diabetic rats was markedly decreased compared with normal rats. Analysis was performed by two-tailed independent Student's *t*-test: * $p <$ 0.01 compared with the normal group ($n = 5$).

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phenomena observed in diabetic rats confirmed the previous report by Rodrigues and co-workers, who determined LPL activity using their radioisotope method. Their results show that LPL activity in the post-heparin plasma is significantly decreased due to hyperglycemia with insufficient secretion of insulin (Rodrigues *et al.*, 1997). These data suggest that hyperglycemia or insulin resistance causes down-regulation activity of circulation LPL. This may account for hypertriglyceridemia, a complication of diabetes. Considering these results together, determination of LPL activity may help us understand the pathological conditions of complications of patients suffering from diabetes mellitus-associated diseases.

In conclusion, we have developed a HPLC method for the highly sensitive determination of circulation LPL activity in 10 μL of rat post-heparin plasma. In addition, we also found that LPL activity in the circulation was drastically decreased in the diabetic stage induced by streptozotocin. The proposed assay offers some advantages as compared with previous reports. First, the most important advantage of this method is that it uses a non-radioactive substrate. This makes the substrate and product separate cleanly with HPLC. Second, our method provides a simple and fast measurement for LPL activity determination. Third, more accurate quantities of products and better qualities of reproduction are guaranteed in our method using margaric acid as an internal standard.

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