

Original paper

Fixed-time and continuous assays of very-low-density lipoprotein secretion rate from rat liver: mean vs. instantaneous velocity

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Abstract

Aim of the study: The secretion rate of triglyceride from rat liver is assayed by the measurement of triglyceride accumulation in plasma when its clearance is inhibited. The aim of the study was to measure and compare the secretion rate of triglyceride from rat liver by two methods of fixed-time and continuous assays.

Material and methods: A single dose of 200 mg of poloxamer-407 (P-407) was injected i.p. into starved male rats. The secretion rate of triglyceride was measured by fixed-time and continuous assays.

Results: The time course for the changes of serum triglyceride following injection of P-407 showed three distinct phases: a lag period of about 30 minutes, a linear increase in serum triglyceride that lasted more than 4 hours, and a slight decline of triglyceride accumulation that lasted about 24 hours. The mean rate of triglyceride secretion was 234.1 ± 9.6 mg/dl/h during the linear phase. The linear phase was divided into five time protocols of 240, 180, 120, 60, and 30 minutes and the secretion rate was measured at three points of time in each protocol. The mean rate of triglyceride secretion was 3.91 ± 0.15 , 3.83 ± 0.16 , 3.76 ± 0.29 , 3.57 ± 0.43 and 3.13 ± 0.34 mg/dl/min in these protocols respectively. In the kinetic assay, the change in the absorbance per three successive five minutes ($\Delta A/\Delta t$) was measured and the secretion rate was calculated as 3.82 ± 0.11 mg/dl/min.

Conclusions: The rate of triglyceride secretion can be measured by both fixed-time and kinetic assays and was about 3.82 ± 0.11 mg/dl/min. The results of the two methods are more corresponded as the mean and instantaneous velocity respectively.

Key words: liver, triglyceride, poloxamer, secretion rate, VLDL

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Introduction

The liver is the major source of plasma triglyceride in the fasted state [1]. Triglyceride is hydrolyzed to glycerol and fatty acids by lipoprotein lipase (LPL) located on the endothelium of capillaries [2]. In any interval of time, the mass of triglyceride secreted from the liver (m_s) is equal to the mass that is hydrolyzed and cleared from the plasma (m_c) plus the mass variation of triglyceride in plasma (m_p) [3]:

$$m_s = m_c + m_p$$

By differentiation of the mass relative to the time as dm/dt , the terms will be changed to rates as follows:

$$dm_s/dt = dm_c/dt + dm_p/dt$$

$$V_s = V_c + V_p$$

This means that the level of triglyceride in plasma is balanced by the rate of the secretion from the liver (V_s) and clearance from the plasma (V_c):

$$V_p = V_s - V_c$$

V_p stands for the changes of plasma triglyceride in a particular time interval (i.e. one hour or minute).

LPL is the rate limiting step in the clearance of triglyceride from the blood stream [2]. Tyloxapol (Triton-1339) and poloxamer (P-407) are nonionic detergents that inhibit LPL completely after a single injection at least for 6 h [4-6]. When the clearance of triglyceride from the plasma is inhibited completely, the term of V_c is equal to zero and the rate of triglyceride secretion will be as it accumulates in plasma:

$$V_c = 0, \quad V_p = V_s$$

This method is applied to estimate the very-low-density lipoprotein (VLDL) associated triglyceride secretion rate from the liver [5-9] and to induce artificial hyperlipidemia [10-12]. The model of experimental lipemia is used to study the effects and mechanism of hypolipidemic drugs [10-12].

There are two protocols to measure the enzyme activity in plasma: fixed-time and continuous monitoring methods [13]. In a typical fixed-time reaction, the enzyme activity is measured by adding 100 μ l of serum to 3 ml of reagent and incubated for a fixed time period, usually 30 minutes. In the kinetic assay, after one minute lag time, the progress of the reaction is usually monitored continuously during the initial three minutes [13]. We developed this idea for the measurement of the secretion rate of triglyceride from the liver. In the classical fixed-time method, the mean secretion rate of triglyceride from rat liver is usually assayed for two, three or four hours [4-9]. The average triglyceride secretion rate from rat liver is about 250 mg/dl/h (or 4.2 mg/dl/min) [14-16]. This high value and improvements in photometric techniques and sampling, leading to more reliable and sensitive measurement, permit us to measure the rate continuously in the short periods. The current study was conducted to measure the secretion rate of triglyceride from rat liver by both methods of fixed-time and continuous monitoring and to compare the results.

Material and methods

Poloxamer (P-407) was obtained from Sigma (USA) and diethyl ether was purchased from Merck. All other chemicals and solvents were of reagent quality and were obtained from local suppliers.

Animals, diets

Thirty-five albino (Wistar) male rats were obtained from the animal center at our university and housed in a room with a 12-h light/dark cycle under constant temperature (25°C) for 15 days. The rats were about 50 days old (weighing 200-220 g) and were fed standard rodent laboratory food and starved overnight (12 h) before blood sampling. All experimental pro-

cedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (GCULA) and approved by ethics committee of our university.

Poloxamer injection

All experiments were commenced at 9.00 a.m. Poloxamer was dissolved in isotonic saline as 20% solution with slight agitation in an ice bath and left standing for a few hours [14]. One milliliter of detergent (1000 mg/kg) was injected intra-peritoneally (i.p.) and the time adjusted as zero 30 minutes thereafter for all experiments.

Venipuncture for fixed-time and kinetic assays

After 30 minutes lag time, the blood samples (200 μ l) were taken from the tail vein at defined times of 0, 15, 30, 60, 120, 180 and 240 minutes under very light ether anesthesia. Blood taking was not successful at all points of time for all rats.

For the kinetic assay, the blood samples were taken from the inferior vena cava above the kidney at 0, 5, 10, 15 minute intervals usually at the time of one hour. We took the blood sample first from the tail vein and then from the inferior vena cava after laparotomy. The absorbance of triglyceride was reduced about 0.010 (less than 5%) by this shifting in the blood sampling, so multiple blood samples were drawn from the vena cava for simplicity and to improve the agreement of the results. After withdrawing the needle, a cotton ball was pushed gently on the site and held to prevent bleeding. If there was bleeding, the blood that entered the peritoneal cavity could not be collected for the measurement of triglyceride. The remaining poloxamer in the cavity inhibits lipase in the kit for triglyceride measurement, so the assay underestimates the value. The blood serum was separated by 5 minutes centrifugation at 3000 rpm and stored at 4°C before analysis or at -70°C for longer preservation.

Measurement of serum lipids

Triglyceride was measured on fresh serum in duplicate by the enzymatic method of GPO-PAP, shaking in a water bath at 37°C for 10 minutes (Pars-Azmon Inc., Tehran).

Statistical analysis

The results are presented as the means \pm standard deviations of the means (SD) of two inter-assays performed in 5-9 different rats at each point of time. To compare the methods, the values of means and stan-

standard deviations were compared by using *t*- and *F*-tests respectively. The percentage coefficient of variation (CV%) is calculated as standard deviation divided per mean. Linear regression was used to show the results of measurements at three points of time. Standard deviations for the slopes of the lines of linear regression (SDr) were calculated as follows:

$$\sqrt{\frac{\sum_i^n (y_i - \bar{y})^2}{n - 2}}$$

using Excel software, in which $(y_i - \bar{y})$ is the error in the predicted value (by regression line) and $n - 2$ is degrees of freedom.

Results

Overall time course for changes of serum triglyceride

The time course for the changes of serum triglyceride following injection of a single dose of P-407 showed three distinct phases (Fig. 1). The basal fasting concentration of triglyceride was 59.6 ± 9.6 mg/dl and after a 30 minutes lag period (first phase) rose linearly during 4 hours tested here (second phase). In the third phase, triglyceride accumulation declined slightly and it reached 2668.3 ± 169.7 mg/dl after 24 hours. The linear portion of Fig. 1A is shown in Fig. 1B to em-

phasize the existence of a lag period of approximately 30 minutes and the linear accumulation of triglyceride up to 4-6 hours. The slope of the line during this phase indicates the mean rate of triglyceride secretion as 234.1 ± 9.6 mg/dl.h. According to Fig. 1, correct results cannot be obtained if the rate is measured during the lag phase (I) or during phase III.

Fixed-time assay of VLDL-triglyceride secretion rate

We divided the linear phase into five time protocols: 240, 180, 120, 60 and 30 minutes (Fig. 2). In any protocol, the blood samples were taken at three points of time and the concentration of triglyceride was measured. Protocols A, B and C are more classically used and the blood samples are taken three times up to 240, 180 and 120 minutes respectively [5-8]. In protocols D and E, the time of sampling was reduced to 60 and 30 minutes. The protocol with the measurements at the first 5, 10 and 15 minutes was not performed because there was a great variation in the lag time between animals. The mean rate of triglyceride secretion was deduced from the equations of the lines as 3.91 ± 0.15 , 3.83 ± 0.16 , 3.76 ± 0.29 , 3.57 ± 0.43 and 3.13 ± 0.34 mg/dl/min in these protocols respectively. It is observed that, as the time interval is decreased from 240 to 30 minutes, the mean rate of triglyceride secretion tends to decrease slightly but not significantly. P-407 in

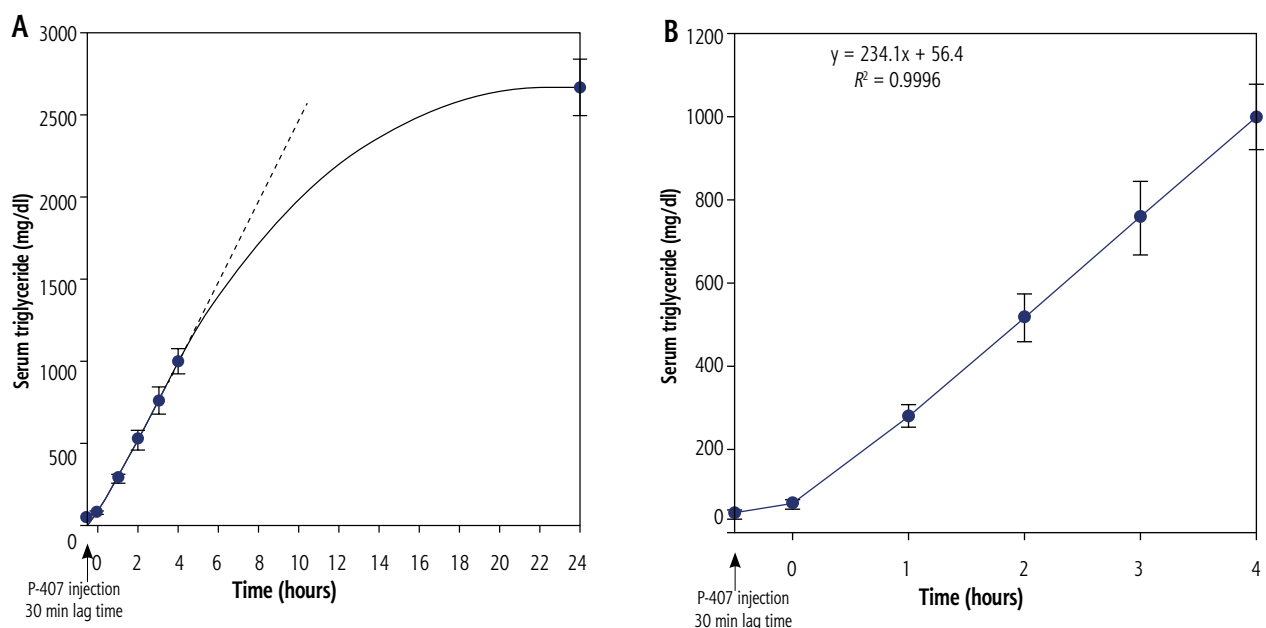


Fig. 1. A) Overall time course for the changes of serum triglyceride following injection of poloxamer. A single dose of 200 mg P-407 was injected by i.p and the zero time adjusted as 30 minutes thereafter. Values are means \pm SD of two inter-assays performed in five up to nine different rats. **B)** Linear region of the changes for serum triglyceride

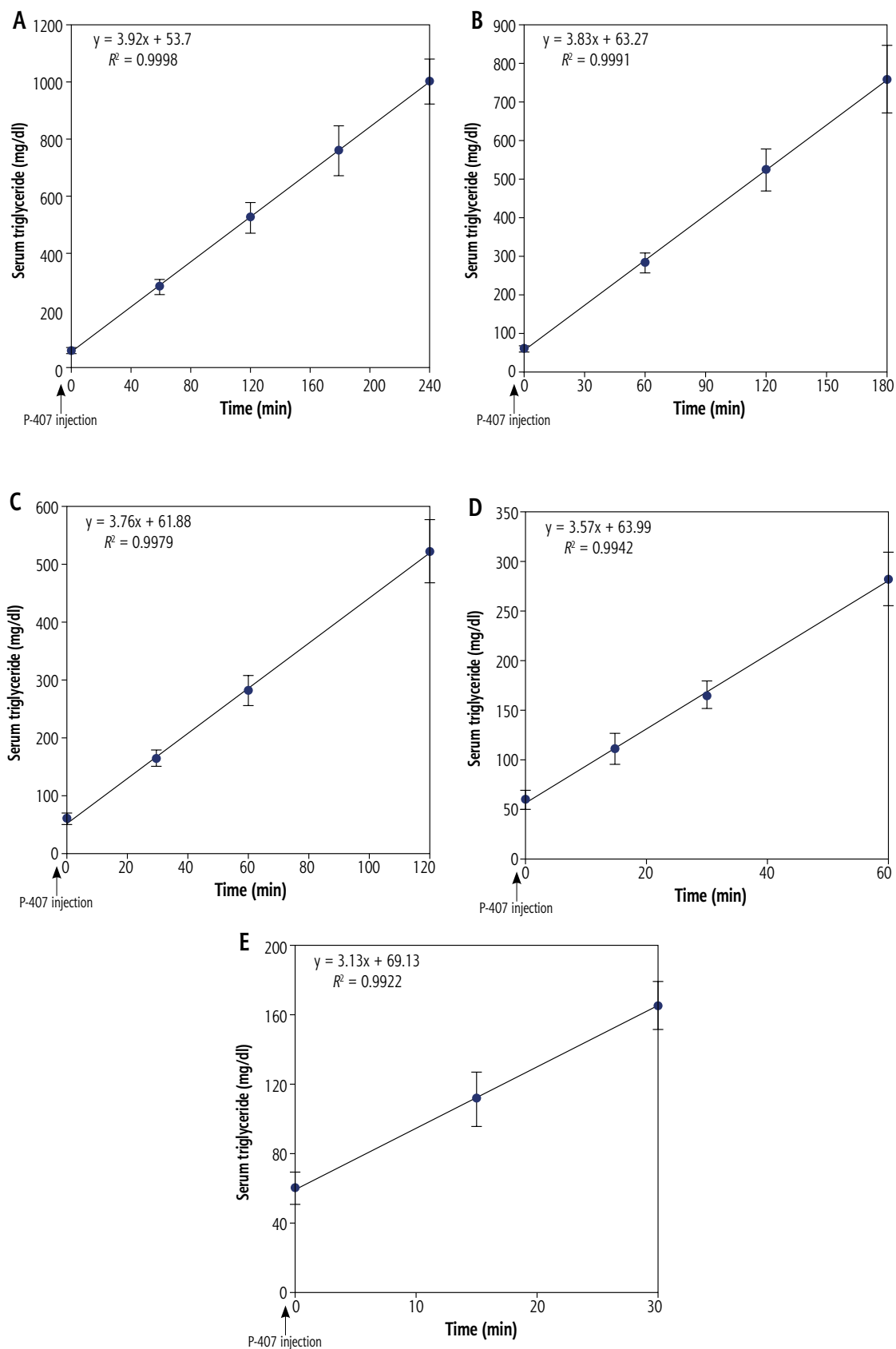


Fig. 2. Fixed-time protocols to measure the mean velocity of triglyceride secretion. A single dose of 200 mg of P-407 was injected i.p. and zero time adjusted as 30 minutes thereafter. Values are means \pm SD of two inter-assays performed in five to nine different rats

Table 1. Continuous assay of VLDL-triglyceride secretion rate. A single dose of 200 mg of P-407 was injected i.p. and zero time adjusted as 30 minutes thereafter. At the time of 1 hour, the peritoneal cavity was opened and another four samples were drawn from the vena cava at 0, 5, 10 and 15 minutes. Values are the means of two inter-assays; the results of any experiment in one separate rat are presented in one row

Basal absorbance at 60 minutes	$\Delta A/\Delta t$, Absorbance/min			$\Delta A/15$	V_s (mg/dl/min)
	$\Delta A_1/5$	$\Delta A_2/5$	$\Delta A_3/5$		
0.254	0.030	0.021	0.022	0.073	3.82
0.302	0.030	0.018	0.027	0.074	3.92
0.258	0.020	0.029	0.022	0.071	3.71

Table 2. Comparison of the results of measurement of triglyceride secretion rate from rat liver by classical fixed-time and kinetic assay methods. In the fixed-time method, the measurement was made at one or three points of time. The values of means and coefficients of variation (CV%) were compared to the first row (single point at 240 min) using *t*- and *F*-tests respectively. SDr stands for standard deviation about the regression

Method	Mean \pm SD (mg/dl/min)	CV%	<i>p</i> -value (<i>t</i> -test)	<i>p</i> -value (<i>F</i> -test)
Classical assay at a single-point	Mean \pm SD			
240 min	3.91 \pm 0.45	11.5%	–	–
180 min	3.88 \pm 0.49	12.5%	0.927	0.522
120 min	3.82 \pm 0.48	12.7%	0.872	0.857
60 min	3.71 \pm 0.43	11.8%	0.608	0.717
30 min	3.53 \pm 0.47	13.2%	0.307	0.791
Classical assay at multiple points	Mean \pm SDr			
240, 180, 120, 60 min	3.91 \pm 0.15	4.0%	0.848	0.019
180, 120, 60 min	3.83 \pm 0.16	4.2%	0.806	0.058
120, 60, 30 min	3.76 \pm 0.29	7.6%	0.547	0.091
60, 30, 15 min	3.57 \pm 0.43	12.4%	0.335	0.175
30, 15 min	3.13 \pm 0.34	10.9%	0.155	0.171
Kinetic assay	Mean \pm SDr			
5, 10, 15 min	3.82 \pm 0.11	2.7%	0.831	0.064

the peritoneal cavity is absorbed gradually and enters the general blood stream, a process that lasts different lengths of time in the rats. Thus, the inhibition of LPL is not complete at the initial period and the measurement of triglyceride secretion is underestimated. For this reason, as long as the time interval is closer to the lag time and compromises it more, the assay of secretion rate will be more underestimated.

Continuous monitoring of VLDL-triglyceride secretion rate

In the kinetic assay of plasma enzymes, the progress of the reaction is monitored continuously, usually at the initial three consecutive one-minute intervals. The changes in the absorbance of our samples were as small as 0.005 per minute, so we chose three successive five-minute intervals for blood sampling. The change in the absorbance in the three next five-minute periods ($\Delta A/\Delta t$) is shown in Table 1 for three rats. In the last column, the secretion rate is presented for different rats as about 3.82 ± 0.11 mg/

dl/min. This value is very similar to the instantaneous velocity and is comparable to the mean velocity obtained by the classical fixed-time assay.

Comparison of fixed-time and kinetic assays

The rate of triglyceride secretion from rat liver was measured by classical fixed-time and kinetic methods, and the results are presented in Table 2. In the fixed-time method, the assay was done at a single or multiple points of time. The values of means and CV% were compared to the values obtained at a single point of 240 minutes (first row) using *t*- and *F*-tests respectively. SDr stands for standard deviation about the regression.

All fixed-time assays performed at a single point had a high CV% of about 10%. In the fixed-time assay, if the measurement was done at three times the CV% will decrease to the acceptable level of 5%, especially for the protocols of 240, 180 and 120 minutes. The decrease in time of the protocols from 240, to 180, 120, 60 and 30 minutes tends to decrease the means and to increase

the CV% slightly. The decrease in the means is not due to analytical error, but is correlated with interference of the lag time and incomplete inhibition of LPL by P-407. The increase in CV% is analytically attributed to the decrease in the absorbance of the samples with lower levels of triglyceride and the variations between animals.

Discussion

The results of the current study show that: 1. If the rate of triglyceride secretion is measured at a single point of fixed time (e.g. at 2 hours) the CV% of the assay will be high. But multiple assays at different times reduce the CV% to about 5%. 2. In the fixed-time method, the time of the assay can be shortened from 240 to 180, 120, 60 and 30 minutes. The reduction of the time was accompanied by a slight decrease of the mean and increase in standard deviation of the method. 3. The rate can be monitored continuously at three successive five-minute intervals at any part of the linear region. This is the first experiment that has applied continuous monitoring to measure the instantaneous rate of triglyceride secretion from rat liver. The rate of triglyceride secretion in starved male rats obtained here using both fixed-time and continuous assays was 3.82 ± 0.11 mg/dl/min, which is comparable to the average value obtained from different references [14-16]. The two methods have different advantages and limitations. The results of the continuous monitoring method are more quickly attainable and closer to instantaneous velocity but are more sensitive to analytical errors.

Instantaneous versus mean secretion rate

Very-low-density lipoprotein is assembled by apolipoprotein-B and the lipid portion, mainly triglyceride, cholesterol and phospholipids [17]. The liver secretes VLDL continuously and the secretion is under acute and chronic regulation. Some factors regulate the secretion acutely, i.e. in seconds and minutes [18-20], while others have long actions on VLDL secretion, i.e. over hours and days [21, 22]. Thus, sometimes it is necessary to measure the secretion rate in a short time. In the fixed-time assay, the mean rate of secretion is measured along the time, usually during three hours. The value obtained by the fixed-time method contains both the first interval, when the P-407 has not entered the blood stream completely, and the last stage, when the inhibitor has entered the liver and directly affects the synthesis and secretion of VLDL [24, 25]. However, in the continuous or kinetic assay it is possible to measure the instantaneous velocity at any point of time. The instantaneous velocity is defined as the limit of ΔC (or ΔA)/ Δt when Δt goes to zero, where

C and A stand for concentration and absorbance respectively. In the kinetic assay of the enzyme activity, the change in absorbance is high enough to measure during the initial three successive one-minute periods. But the change in the absorbance of the samples in this study was low, so it is hard to see significant and reproducible change in a shorter period. In the kit that we used to measure triglyceride the ratio of standard concentration to absorbance was 200 (mg/dl)/ 0.250 , but some kits have higher sensitivity. So it is possible to monitor the rate of secretion at a shorter time such as three successive 2.5-minute periods, if such kits are used. In general, it is preferred to shorten the time of the assay. However, analytically it causes the standard deviation and CV% of the method to increase. Such changes can be avoided by measuring the rate at multiple points of time (Table 2). By using multiple assays, SD is substituted by SD of regression (SDr) and the results will be improved.

Initial velocity is attainable just following i.v. injection

Tyloxapol, another LPL inhibitor, is usually injected intravenously (i.v.) and after 5 minutes lag time causes complete inhibition of triglyceride clearance [8]. Therefore, it is possible to measure the initial velocity of triglyceride secretion in the first minutes following i.v. injection of tyloxapol. However, poloxamer is injected i.p. and there is a variable lag time of about 30 minutes to enter the blood stream and inhibit LPL completely. This uncertainty in the duration of the lag time can be seen in the initial level of triglyceride after i.p. injection in different references [26, 27]. So, initial velocity cannot be measured correctly if P-407 is administered i.p.

Nutrition and anesthesia are important interfering factors

There is a standard dried food for animals, but usually it is deficient in fresh vitamins, minerals, carbohydrates, lipids and proteins. So we observed that in the rats with standard chow the level of glycogen in the liver was near to zero even in a fed state and the plasma triglyceride did not increase after the injection of LPL inhibitor [18, 19]. Malnutrition impairs triglyceride secretion by up to 90%, especially after one hour following the injection of LPL inhibitor (results not shown) [4]. Thus the rats must have required nutrition during the week preceding the experiment to show normal metabolism such as VLDL secretion.

Anesthesia with ether is the second important factor that may influence the rate of triglyceride secretion. As reported previously, repeat exposure to ether can

reduce the rate of secretion by up to 35% [4]. This effect of ether is only significant when it is repeated at a short interval of less than 30 minutes but not more than 60 minutes. To prevent such an effect, we used very light anesthesia, the blood was taken rapidly and the rats were used only at one point of time.

Conclusions

The rate of triglyceride secretion can be measured by both fixed-time and continuous assays. The results of the two methods are more corresponded as the mean and instantaneous velocity respectively. The rate of triglyceride secretion obtained by both methods was about 3.82 ± 0.11 mg/dl/min.

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Disclosure

The authors declare no conflict of interest.

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