

Plasma phosphatidylcholine hydroperoxide as a new marker of oxidative stress in alcoholic patients

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Running title: Plasma Phosphatidylcholine Hydroperoxide in alcoholics

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHT, 3,5-di-tert-butyl-4-hydroxytoluene; CL, chemiluminescent detection; γ -GTP, γ -glutamyl transpeptidase; TC, total cholesterol; TG, triglyceride; PCOOH, phosphatidylcholine hydroperoxide.

Abstract Quantitative analysis of plasma phosphatidylcholine hydroperoxide (PCOOH) is an important step to evaluate the biochemical processes leading to oxidative injury. However, secondary products of lipid peroxidation are now used as indices. One hundred and nine alcoholic patients, aged 22-81 years (mean±SE, 52.0±1.3 years), and 21 healthy volunteers, aged 41-79 years (51.2±2.2 years) participated in this study. Plasma PCOOH was measured by HPLC with chemiluminescent detection. Plasma PCOOH concentration was significantly higher in alcoholic patients (46.1±4.1 pmol/ml) than controls (15.6±1.8 pmol/ml). It was significantly higher in patients with blood alcohol (88.0±10.5 pmol/ml) than those with non-alcohol (32.6±3.1 pmol/ml). The patients with high levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transpeptidase (γ -GTP), and triglyceride (TG) showed significantly higher PCOOH concentration than normal levels of them. The PCOOH level was positively correlated with levels of γ -GTP, HDL, blood alcohol concentration, and TG. Plasma PCOOH levels in 29 alcoholic patients after a six-week abstinence were decreased significantly (22.8±11.1 pmol/ml), which was associated with improvement of liver function test. This is the first measurement of plasma PCOOH in alcoholic patients. These results suggest the involvement of lipid peroxidation in alcohol-induced liver damage. These findings confirm that the PCOOH plasma concentration is a new marker of alcohol consumption as well as oxidative stress in alcoholic patients.

Supplementary key words phosphatidylcholine hydroperoxide, plasma, alcoholic patients

INTRODUCTION

The induction of CYP2E1 in hepatocyte (1, 2) as well as in Kupffer cells (3) by chronic alcohol has been shown to generate free radicals and cause liver disease due to peroxidation of membrane lipids.

Previously, we developed methods for quantifying cholesterol hydroperoxides by HPLC with chemiluminescent detection (HPLC-CL) (4) and oxysterols by HPLC with UV detection (5). Subsequently, we demonstrated elevated membrane cholesterol peroxidation by analyzing 7-hydroperoxycholesterols as well as oxysterols in skeletal muscles (6), liver (7), and heart (5) of rats with chronic administration of alcohol. However, direct demonstration of increased lipid peroxidation in patients with alcoholic liver disease (ALD) has been difficult because plasma concentrations of 7-hydroperoxycholesterol and oxysterols are too low to be detected. Recently lipid peroxidation has been assessed by analyses of 4-hydroperoxynonenal (8), serum malondialdehyde (9, 10), and F₂-isoprostane excretion (11), secondary products of phospholipid peroxidation. Plasma phosphatidylcholine hydroperoxide (PCOOH), a direct peroxidation product, may be a sensitive and specific index of lipid peroxidation in vivo.

To assess oxidative stress in alcoholic patients with ALD, PCOOH plasma concentration was measured in 109 alcoholic patients and 21 healthy controls. Additionally, factors relevant to the lipid peroxidation among various clinical tests were investigated in alcoholic patients.

MATERIALS AND METHODS

Materials

3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT), luminol (3-aminophthaloylhydrazine), and cytochrome C (from horse heart, type VI) were purchased from Wako Pure Chemical Co. (Osaka, Japan). 1-Palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide (C16:0/C18:2-

OOH) was synthesized by reaction with methylene blue under tungsten lamp irradiation at 15°C for 8 h, monitoring by HPLC and TLC. The whole reaction mixture was subjected to HPLC (Daiso-gel, SP-120-40/60-ODS-B, CHCl₃- methanol, UV 235 nm, at 50 ml/min). The product obtained was checked by MS, TLC, and HPLC. Additionally phosphine amount was quantitatively analyzed as 1.95 mg/ml (MS: 790.6 (M+H, monoperoxide) and 812.5 (M+Na, monoperoxide)).

Subjects

One hundred and nine alcoholic patients, 93 men and 16 women, 22 to 81 years old, (52.0±1.3 years) were used, while twenty-one healthy volunteers, 20 men and one woman, 41 to 79 years old (51.2±2.2 years), were recruited after obtaining their informed consents. This experiment had the approval of the ethical committee of National Institute on Alcoholism, Kurihama National Hospital. The investigation conforms to the principles outlined in the Declaration of Helsinki. Blood samples were collected from alcoholic patients at the first medical examination and after six-week abstinence.

Serum transaminase (AST and ALT) activities, γ -glutamyl transpeptidase activity (γ -GTP), and the levels of serum albumin, cholinesterase, total bilirubin, total cholesterol (TC), triglyceride (TG), HDL, and creatinine were examined using routine laboratory techniques.

Six-weeks after hospitalization the level of Type 4 collagen, and anti-hepatitis C virus antibody of 29 patients were determined. Of these, we diagnosed 9 patients with alcoholic hepatitis and 11 with liver cirrhosis using ultrasonography and laboratory data.

Extraction

Three millilitres of blood was collected in a test tube containing 0.3 mg of EDTA.2Na, centrifuged at 4°C and 800×g for 10 min and the plasma fractionated. Total lipid was extracted by adding 0.5 ml of distilled water and 8 ml of ice-cold chloroform/methanol (3:1, v/v), containing 0.005 % (v/v) BHT (as antioxidant) to 0.5 ml of plasma. The mixture was spun vigorously for 1 min then centrifuged at 800×g for 20 min. The chloroform layer was aspirated off and concentrated in a rotary evaporator, then dried under a nitrogen stream. The phospholipid fraction then was isolated from the total lipid by solid phase extraction. A silica column (Sep-Pak, Waters Co. Milford, MA) of 3 ml capacity packed with aminopropyl-derivatized silica (-NH₂) initially was conditioned by washing with 5 ml of acetone and 10 ml of n-hexane. The total lipid sample, dissolved in a small amount of chloroform, was layered on the column which then was flushed with a mixture of 2 ml chloroform and 1 ml iso-propanol, giving an eluate consisting mainly of cholesterol. The column next was flushed with methanol containing 0.005% BHT, giving an eluate consisting mainly of phospholipid. This was concentrated in a rotary evaporator, dried under a nitrogen stream, then dissolved in 150 µl methanol, and a 10 µl portion was injected into the HPLC column.

HPLC-CL Analysis

PCOOH was analyzed by HPLC-CL. The apparatus consisted of two LC-10AD vp pumps (Shimadzu, Kyoto, Japan), a CLD-10A chemiluminescence detector (Shimadzu), and a Chromatopac C-R8A integrator (Shimadzu).

An LC-18-DB column (SUPELCO, 250 × 4.6 mm internal diameter) with methanol containing 0.01 % triethylamine as the mobile phase was used. Both the mobile phase and the chemiluminescent reagent were delivered at 0.7 ml/min. The reagent consisted of cytochrome C and luminol (10 and 2 µg/ml, respectively) in alkaline borate buffer, pH10. After the column

eluant was passed through a UV detector set at 234 nm, it was mixed with the luminescent reagent in the post-column mixing joint of the chemiluminescence detector.

Individual peak areas were calculated with an integrator (Chromatopac C-R8A, Shimadzu). Standard PCOOH was injected at least three times a day to calculate the concentration, because chemiluminescent intensity was sometime unstable particularly during the early period of analysis. The recoveries from the plasma extracts were determined by comparison of the peak area obtained after injection of a plasma extract spiked with a known concentration. The recoveries were about 70%.

Statistical Analysis

All data are presented as means \pm standard error (SE). Differences between two groups were assessed using Students' *t*-test. Step-wise multiple regression analysis (SMR) was performed to provide a simultaneous model of prediction.

RESULTS

Typical HPLC-CL chromatograms for PCOOH are shown in **Fig. 1**, which illustrate the chromatographic separation of PCOOH in a standard solution, plasma samples from the control, and the alcoholic subjects. Standard PCOOH gave a single peak at 7.82 min. The plasma extract had peaks 1 at 7.81 and 2 at 9.36 min. Accordingly, the retention time of peak 1 corresponded to that of standard PCOOH.

Blood alcohol was detected in 27 alcoholic patients at the first medical examination. Of these, half of the patients had blood alcohol concentration between 0.1 and 1 mg/ml (**Table 1**).

Comparison of the PCOOH concentration in alcoholic and control subjects showed that the PCOOH concentration was significantly higher in alcoholics (**Table 2**). The mean concentration in alcoholics was 46.1 ± 4.1 pmol/ml, while in controls it was 15.6 ± 1.8 pmol/ml. PCOOH concentrations in alcoholic patients for groups of blood alcohol, liver function, and lipid metabolism are shown in **Tables 3-5**. The PCOOH concentration was significantly higher in patients with blood alcohol (88.0 ± 10.5 pmol/ml) than without blood alcohol (32.6 ± 3.1 pmol/ml) at the first medical examination (**Table 3**). Thus, blood alcohol evidently affected the accumulation of PCOOH in plasma. Comparison of the PCOOH concentration in alcoholic patients showed that the PCOOH concentrations were significantly higher in patients with high levels of AST, ALT, and γ -GTP (**Table 4**). Thus, the accumulation of plasma PCOOH was affected by clinical tests related to liver function. Moreover, the PCOOH concentration was significantly higher in patients with a high level of TG, whereas it was not higher with a high level of TC (**Table 5**). Statistical Analysis System (SAS) revealed that γ -GTP, HDL, blood alcohol concentration, and TG correlated positively and independently with plasma PCOOH concentration (**Table 6**). Thus, these four biochemical factors have significant influences on elevated PCOOH. In contrast, time between blood collection and the final drink did not correlate with plasma PCOOH concentration by SAS.

Figure 2 shows plasma PCOOH concentrations in 29 patients on admission and after the six-week abstinence. The mean concentration (41.8 ± 6.7 pmol/ml) decreased to almost control value (22.8 ± 2.0 pmol/ml), except one case. The biochemical profiles of this case were practically normal on admission, whereas the fasting blood sugar after the six-week abstinence increased to 150 mg/dl.

Table 7 shows the PCOOH concentrations in patients with alcoholic liver disease. The patients with liver cirrhosis had significantly higher Type 4 collagen and lower choline

esterase activity, but significantly lower PCOOH concentrations than patients with alcoholic hepatitis.

DISCUSSION

The major findings of this study were [1] the plasma PCOOH concentration was significantly higher in alcoholic patients than controls, [2] the PCOOH level positively correlated with levels of γ -GTP, HDL, blood alcohol concentration, and TG, [3] and the elevated PCOOH concentration returned to normal after the six-week abstinence.

As phosphatidylcholine is easier to be peroxidized to produce PCOOH than cholesterol to cholesterol hydroperoxide, plasma PCOOH is easily detectable, whereas cholesterol hydroperoxide is not. Accordingly, plasma PCOOH may be used as a general indicator of lipid peroxidation. Indeed, accumulation of plasma PCOOH was observed not only in dialysis patients with diabetic nephropathy (12), but in patients with hyperlipidemia (13); this may be related to the development and progression of atherosclerosis. In addition, increase of serum PCOOH was dependent on glycemic control in type 2 diabetic patients (14). The effects of hemodialysis in patients with uremia (15) and tea catechin supplementation in healthy volunteers on antioxidant capacity (16) were estimated by plasma PCOOH.

As the main component (peak 1 in **Fig. 1**) of plasma phosphatidylcholine hydroperoxide is PC C16:0/C18:2-OOH, we used it as a standard compound. We were able to identify it and quantify the accurate concentration of it in the present study. The remainder shown as peak 2 in **Fig. 1** may be PC C18:0/C18:2-OOH. We estimate the concentration of peak 2 by calculating peak area based on PC C16:0/C18:2-OOH. The mean area ratio of PC C18:0/C18:2-OOH to PC C16:0/C18:2-OOH was 0.22, almost constant; accordingly total PCOOH concentration may be 1.22 times higher than PC C16:0/C18:2-OOH alone.

Although various serum markers, such as γ -GTP (17), carbohydrate-deficient transferin (17, 18), metalloproteinase-9 (19, 20), tissue polypeptide specific antigen (21), Type 4 collagen (22), laminin (20), hyaluronic acid (23) and CYP2E1 activity (24), have been introduced for the evaluation of alcohol consumption and alcoholic liver disease, it has remained unclear which is the best marker.

To understand the significance of elevated plasma PCOOH concentration in this study, factors relevant to the PCOOH concentration were studied using data collected from alcoholic patients at the first medical examination and after 6 week abstinence.

Firstly, the relationship between blood alcohol and the PCOOH concentrations was examined. As expected, the PCOOH concentration was significantly higher in patients with blood alcohol than without blood alcohol, indicating the evidence of increased oxidative stress due to the direct action of alcohol.

Next, γ -GTP, AST, and ALT are hepatic enzymes that leak from hepatocytes into the blood when hepatocytes are damaged by various causes including alcohol consumption. In these studies, the PCOOH plasma concentrations significantly increased in patients with higher activity of these enzymes, correlating with γ -GTP activity, indicative of elevated oxidative stress in alcoholic liver injury.

The PCOOH concentration significantly increased in patients with high level of TG, which may be caused by decreased activity of lipoprotein lipase, hepatic triglyceride lipase, (25) and long-term alcohol consumption.

The mean PCOOH concentrations decreased in 29 alcoholic patients after the six-week abstinence, showing that PCOOH is a marker of alcohol consumption.

Finally, accumulation of 7-hydroperoxycholestreols was observed in alcoholic fatty liver but not alcoholic cirrhotic liver (26). The present results, showing elevated plasma PCOOH in

patients with alcoholic hepatitis, but low levels in patients with alcoholic liver cirrhosis, consistent with previous liver data, suggest increased lipid peroxidation in the early stage of alcoholic liver injury.

In conclusion, the present findings confirm that plasma PCOOH is a new marker of alcohol consumption as well as oxidative stress in alcoholic patients.

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LEGENDS

Fig. 1. HPLC analyses of standard phosphatidylcholine hydroperoxide (PCOOH) and plasma samples of control and an alcoholic patient by chemiluminescence detection.

A. PC (C16:0/C18:2-OOH); B. Control; C. Alcoholic patient.

Fig. 2. Plasma PCOOH concentrations in 29 patients on admission and after six-week abstinence.

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**TABLE 1. Blood alcohol concentration (BAC, mg/ml)
at the first medical examinations**

Group	n
BAC=0	81
$0 < \text{BAC} \leq 0.1$	3
$0.1 < \text{BAC} \leq 1$	14
$1 < \text{BAC} \leq 2$	7
$2 < \text{BAC}$	3

TABLE 2. Plasma PCOOH concentrations in alcoholic and control subjects

Group	n	Age	PCOOH pmol/ml	p
Alcoholic	109	52.0 ± 1.3	46.1 ± 4.1	
Control	21	51.2 ± 2.2	15.6 ± 1.8	0.0001

Values are means ± SE.

PCOOH, phosphatidylcholine hydroperoxide.

**TABLE 3. Blood alcohol and plasma PCOOH concentrations
at the first medical examination**

Group	n	PCOOH pmol/ml	p
BAC>0	27	88.0 ± 10.5	
<u>BAC=0</u>	81	32.6 ± 3.1	0.0001

Values are means ± SE.

PCOOH, phosphatidylcholine hydroperoxide;

BAC, blood alcohol concentration.

TABLE 4. PCOOH concentrations for biochemical variables related to liver function

Variable		Range	n	PCOOH pmol/ml	p
AST	high	40 IU/L ≤	65	59.3 ± 6.2	0.0001
	normal	40 IU/L >	44	26.6 ± 2.7	
ALT	high	42 IU/L ≤	37	66.3 ± 8.8	0.0027
	normal	42 IU/L >	72	35.7 ± 3.9	
γ-GTP	high	72 IU/L ≤	77	53.2 ± 5.5	0.0003
	normal	72 IU/L >	32	28.9 ± 3.2	

Values are means ± SE.

PCOOH, phosphatidylcholine hydroperoxide; AST, aspartate aminotransferase;

ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase.

TABLE 5. PCOOH concentrations and lipid metabolism

Variable		Range	n	PCOOH pmol/ml	p
TG	high	151 mg/dl \leq	36	67.5 \pm 9.3	0.002
	normal	151 mg/dl $>$	73	35.6 \pm 3.6	
TC	high	220 mg/dl $<$	37	51.8 \pm 7.8	0.331
	normal	220 mg/dl \geq	72	43.2 \pm 4.8	

Values are means \pm SE.

PCOOH, phosphatidylcholine hydroperoxide;

TG, triglyceride; TC, total cholesterol.

**TABLE 6. Correlation between PCOOH concentration
and clinical tests**

Variable	Regression coefficient (%) (95% CI)	p Value
γ -GTP	0.02966 (0.0136-0.046)	0.0005
HDL	0.26045 (0.081-0.440)	0.0054
BAC	8.58888 (0.183-16.994)	0.0478
TG	0.06516 (0.041-0.089)	<0.0001

The multiple adjusted regression coefficient was R=0.613.
CI, confidence interval.

TABLE 7. PCOOH concentrations in patients with alcoholic liver disease

	n	On admission					After 6-weeks
		PCOOH	AST	γ -GTP	ChE	TC	Type 4 collagen
		pmol/ml	IU/L	IU/L	IU/L	mg/dl	ng/ml
Alcoholic hepatitis	9	66.0 ± 13.7	104.2 ± 30.6	433.6 ± 137.2	160.7 ± 14.1	234.4 ± 26.1	8.0 ± 1.0
Liver cirrhosis	10	21.6 ± 5.8	84.2 ± 21.1	346.5 ± 136.6	100.3 ± 13.6	152.2 ± 19.1	16.1 ± 1.8
p		0.006	0.591	0.659	0.006	0.019	0.001

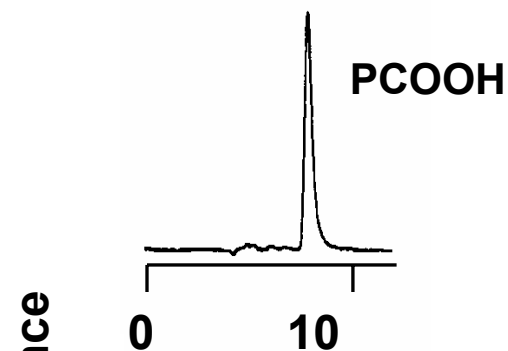
Values are mean \pm SE.

PCOOH, phosphatidylcholine hydroperoxide; AST, aspartate aminotransferase;

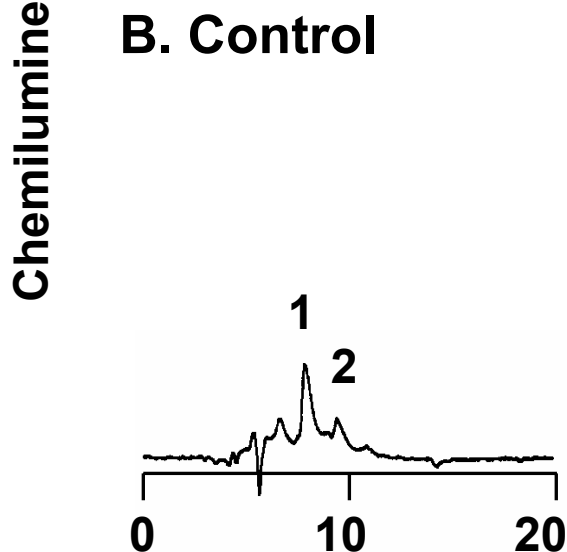
γ -GTP, γ -glutamyl transpeptidase; ChE, choline esterase; TC, total cholesterol.

HPLC Chromatograms of PCOOH

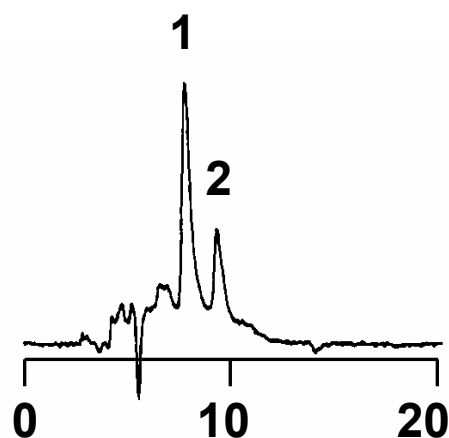
A. Standard



B. Control



C. Alcoholic



Retention time (min)

Fig. 1. HPLC analyses of standard phosphatidylcholine hydroperoxide (PCOOH) and plasma samples of control and an alcoholic patient by chemiluminescence detection.

A. PC (C16:0/C18:2-OOH); B. Control; C. Alcoholic patient.

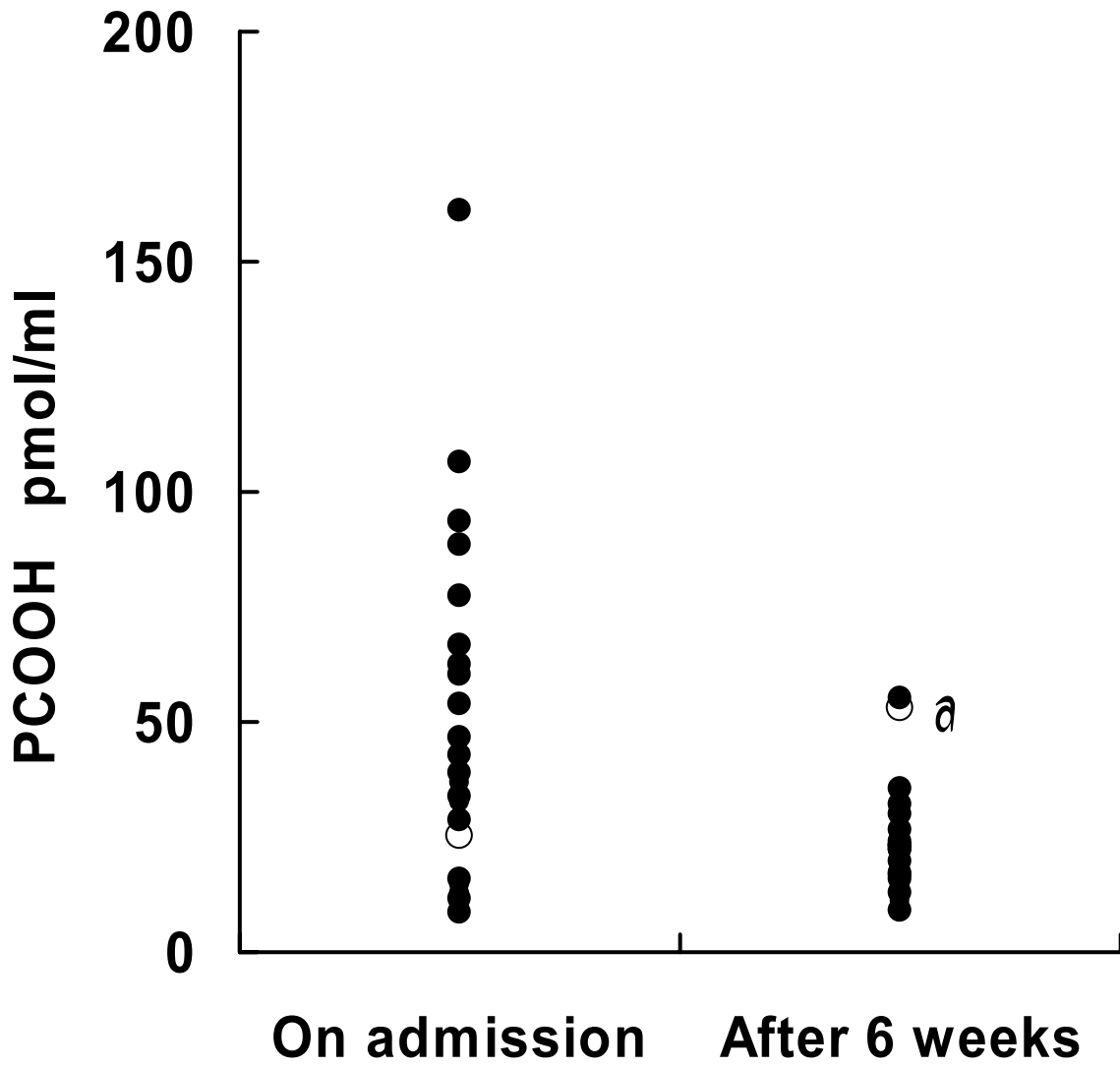


Fig. 2. Plasma PCOOH concentrations in 29 patients on admission and after a 6-wk abstinence.