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# Improved Method for the Determination of Phospholipase $A_2$ Catalytic Activity Concentration in Human Serum and Ascites

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**Summary:** An improved radiochemical method is presented for the selective determination of phospholipase $A_2$ activity in human serum and ascites, using only commercially available reagents. The method can be applied to large quantities of samples. As substrate we used 1,2-dipalmitoyl-sn-glycero(3)phosphorylcholine and phosphatidylcholine containing tritiated palmitic acid in position 2 (1-palmitoyl,2-[9,10-$^3$H]palmitoyl-sn-glycero(3)phosphorylcholine). The liberated fatty acids are extracted and radioactivity is detected in a liquid scintillation spectrometer.

A preliminary reference range of human serum samples was established ranging up to 1.0 U/l. In sera of patients with acute pancreatitis we found activities up to 20 U/l. The correlation of phospholipase $A_2$ activity with that of other enzymes and with the severity and complications of acute pancreatitis was investigated. A possible relationship between phospholipase $A_2$ activities and pulmonary complications is discussed.

## Introduction

The severe course of acute pancreatitis is called necrotizing pancreatitis, derived from the development of the necrosis of the gland itself and of the surrounding tissues. This can lead to shock and multiple organ failure via the liberation of vasoactive substances and toxins (1), resulting in a high mortality (2).

Recently, it was proposed that phospholipase $A_2$ (E. C. 3.1.1.4) is the main agent among the pancreatic enzymes, leading to local necrosis as well as to acute lung failure, due to its destructive action on biomembranes (3).

Phospholipase A activity is elevated in the sera of patients with acute pancreatitis (4). In contrast to the pancreatic enzymes isoamylase, lipase and trypsin (5), an elevation of the phospholipase A activity seems to be connected with a more severe and more complicated course of the disease (6, 7). These methods, however, do not distinguish between phospholipase $A_1$, phospholipase $A_1$-like and phospholipase $A_2$ activity.

In this study, a practicable radiochemical assay for the selective determination of phospholipase $A_2$ is presented, which is based on the procedure of *Shakir* (8), using only commercially available reagents. It is applied to patients morphologically well defined by computed tomography and/or intraoperative findings (9).

## Materials and Methods

### Reagents and Instruments

*Reagents for the phospholipase $A_2$ assay*

1,2-dipalmitoyl-sn-glycero(3)phosphorylcholine, (substrate) referred to hereafter as phosphatidylcholine (Serva, Heidelberg, FRG); 2-[9,10-$^3$H]palmitoyl, 1-palmitoyl-sn-glycero(3)phosphorylcholine and [1-$^{14}$C]palmitic acid (NEN, Dreieich, FRG); sodium deoxycholate (Fluka, Neu-Ulm, FRG); purified phospholipase $A_2$ from hog pancreas (Boehringer, Mannheim, FRG); 2,5-diphenyloxazole (PPO) (Zinsser, Frankfurt, FRG); 2,2'-*p*-phenylenebis(5-phenyloxazole) (POPOP) and all other reagents (Merck, Darmstadt, FRG).

*Instruments*

Multipette (Eppendorf, Hamburg, FRG); dispenser (Boehringer, Mannheim, FRG); membrane pump (Braun, Melsungen, FRG) for the silicic acid/*n*-heptane suspension, Liquid Scintillation Spectrometer LS 1801 (Beckman, Dreieich, FRG).

Pancreas isoamylase was measured according to l. c. (10), using the Phadebas Isoamylase test kit (Pharmacia, Uppsala, Sweden). Immunoreactive pancreatic lipase was measured using Enzygnost Lipase (11) (Behring, Frankfurt, FRG).

## Samples

Serum and ascites of clinically well evaluated patients with acute pancreatitis were stored deep frozen up to 4 months until analysis. The samples were centrifuged after thawing to remove particles. The presence of pancreatitis was ascertained from clinical, laboratory and morphological findings.

## Method

A 12 mmol/l solution of phosphatidylcholine in chloroform/methanol (2 vol + 1 vol) was prepared. Tracer was added until 0.05 ml of this solution contained 20 000 counts/min. This stock solution was stable for several weeks at −28 °C. An aqueous 20 mmol/l glycine buffer containing 6 mmol/l sodium deoxycholate was adjusted to pH 8.0 using 1 mol/l NaOH. To prepare a substrate solution, the stock solution was dried in a water bath at 40 °C under a stream of nitrogen, and dissolved again in the double volume of buffer to attain a substrate concentration of 6 mmol/l. It was mixed well, heated for one minute to 60 °C and cooled down to room temperature. To each 10 ml of this mixture 0.1 ml of a 0.2 mol/l aqueous $CaCl_2$ solution was added to obtain a clear solution. This substrate solution was prepared afresh for each assay procedure.

In each experiment three 0.02 ml aliquots of serum or ascites samples were pipetted into PPN (polypropylene) vials. Purified hog pancreas phospholipase $A_2$, diluted 1 : 250 000 (vol/vol) in an aqueous solution of bovine serum albumin (60 g/l) and NaCl (9 g/l) as a control sample and the same bovine serum albumin/NaCl solution as a blank sample, were pipetted into PPN vials in triplicates. To each vial 0.1 ml substrate solution, containing 600 nmol phosphatidylcholine, were added. To determine the total radioactivity of the labelled phosphatidylcholine, two 0.1 ml aliquots of the substrate solution were pipetted into scintillation vials and stored at room temperature until liquid scintillation counting. After incubation for 180 minutes in a water bath at 40 °C the reaction was stopped by adding 0.1 ml aliquots of *Dole*'s reagent (12) (isopropanol/*n*-heptane/sulphuric acid (1 mol/l) = 4 vol + 1 vol + 0.025 vol).

All subsequent steps were performed at room temperature. After ten minutes, 1 ml aliquots of a silicic acid/*n*-heptane suspension (10 g/l) were added and mixed thoroughly for 30 seconds. To absorb the phosphatidylcholine contaminating the upper phase, again 1 ml aliquots of a $SiO_2$/*n*-heptane suspension (20 g/l) were added. It was necessary to mix the suspensions continuously during pipetting. The suspension was prepared from precipitated dried silicic acid, with a particle size less than 0.1 mm. An additional heat activation of the silicic acid was not necessary.

After ten minutes the samples were centrifuged for a few seconds at 1000 *g* and decanted into scintillation vials. 2 ml *n*-heptane were added to the two scintillation vials containing 0.1 ml radioactive substrate solution. 8 ml scintillation cocktail (5.5 g PPO, 0.07 g POPOP, 667 ml xylene, 333 ml Triton X 100) were added to each vial, mixed and measured in a liquid scintillation counter.

The catalytic activity concentrations *b* of the samples were calculated:

$$b = \frac{\text{counts/min (sample)} - \text{counts/min (blank)}}{\text{counts/min (total activity)}} \cdot \text{factor}$$

$$\text{Factor} = \frac{600 \cdot 10^{-3}\ \mu\text{mol}}{0.8 \cdot 180\ \text{min} \cdot 20 \cdot 10^{-6}\ \text{l}} = 208.3\ \text{U/l}$$

1 U = 1 micromol liberated palmitate per minute

The factor was composed of the amount of substrate added per vial (600 nmol), the recovery index for the liberated fatty acid (0.8) the incubation time (180 min) and the sample volume (0.02 ml).

## Results

The kinetics of the reaction of phospholipase $A_2$ are shown in figure 1 for a blank sample (a), for the control sample of purified phospholipase $A_2$ (b) and for sera of patients with acute pancreatitis (c−g). The reaction shows a linear response over at least 180 minutes. In the blank sample, which was human serum incubated after addition of some crystals of $Na_2$-EDTA to trap the calcium-ions, we found only a minimal pseudoactivity of 0.1 U/l originating from the spontaneous hydrolysis of phosphatidylcholine. The spontaneous hydrolysis and radiolysis of phosphatidylcholine in EDTA-inhibited serum samples was in the same range as in the albumin blank (not shown). This indicates that there is no additional spontaneous hydrolysis in serum.

In a dilution experiment (fig. 2) we used purified hog pancreas phospholipase $A_2$ dissolved and diluted in the serum of a healthy person (a). An ascites sample of a patient with acute pancreatitis was diluted in bovine serum albumin solution (b). The blank was serum or albumin solution devoid of purified phospholipase $A_2$. We obtained linearity up to at least 30 U/l.

An optimal substrate concentration was evaluated using an ascites sample of a patient with severe acute pancreatitis. Phospholipase $A_2$ activity was measured employing phosphatidylcholine concentrations ranging from 0.1 to 12.5 mmol/l in the assay mixture at a constant ratio of unlabelled to labelled substrate (fig. 3, curve a). Substrate concentrations higher than 12.5 mmol/l were not employed due to solubility problems, and maximal velocity ($V_{max}$) was not reached at the highest experimental substrate concentrations. As indicated in curve b, we found a maximal yield between 3 and 8 mmol/l. At a substrate concentration of 5 mmol/l optimal assay conditions were reached with respect to accuracy and turnover of the labelled substrate.

The simplification of the extraction procedure resulted in an improvement of the fatty acid recovery rate. It was almost twice as high (79%), as that obtained using the original method of *Shakir* (49%).

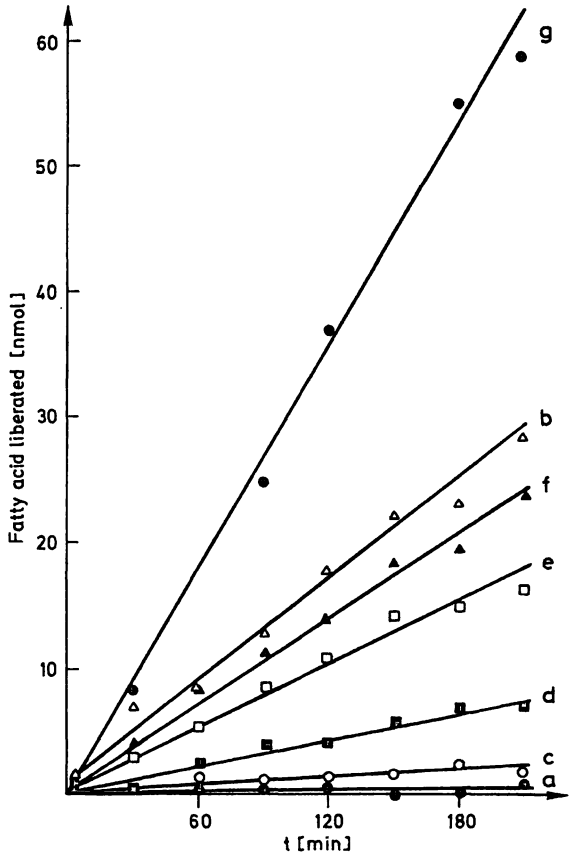


Fig. 1. Liberation of labelled palmitic acid monitored during an incubation time of 210 minutes; the first blank value was subtracted from all other values.
a) Blank sample of human serum and additional $Na_2$-EDTA.
b) Standard sample of hog pancreas phospholipase $A_2$, diluted 1 : 250 000 (vol/vol).
c–g) sera of patients with acute pancreatitis.

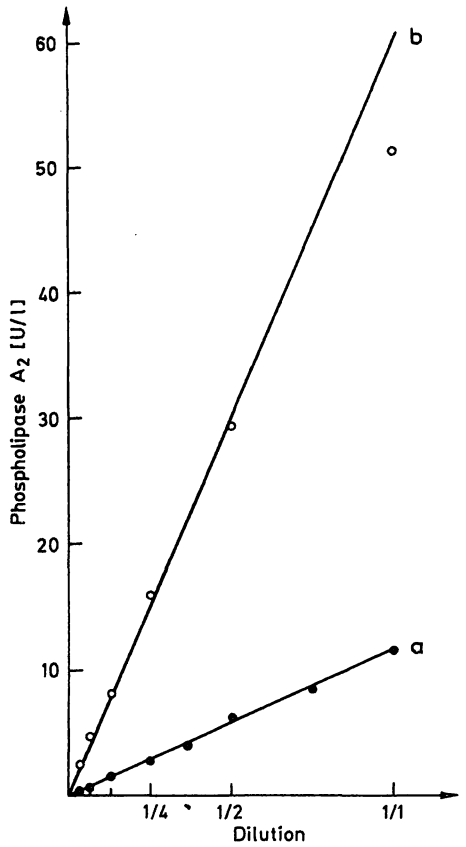


Fig. 2. Dilution of phospholipase $A_2$-containing samples;
a) Purified phospholipase $A_2$ from hog pancreas in a stock solution (1 : 125 000), diluted in serum of a healthy person.
b) Ascites sample of a patient with acute necrotizing pancreatitis, diluted geometrically in bovine serum albumin solution.

The two-step addition of the $SiO_2$/*n*-heptane suspension led to 80% extraction of the fatty acids from the aqueous into the *n*-heptane phase, and a thorough adsorption of the traces of phosphatidylcholine was achieved. Without the second step, the separation of fatty acid and phosphatidylcholine was inadequate (not shown). In the blank sample we found a spontaneous radiolysis and hydrolysis in the range of 1% to 1.5% of the employed substrate.

The interassay and intraassay precision, obtained with the control sample (diluted hog pancreas phospholipase $A_2$) was below 10% and 5%, respectively (tab. 1).

Tab. 1. Precision of the phospholipase $A_2$ assay. The intraassay variability was calculated from single measurements, the interassay precision from means of triplicates.

| Precision | n | Mean (U/l) | SD (U/l) | CV (%) |
|---|---|---|---|---|
| Interassay | 6 | 9.1 | 0.8 | 8.8 |
| Intraassay | 10 | 10.2 | 0.4 | 4.1 |

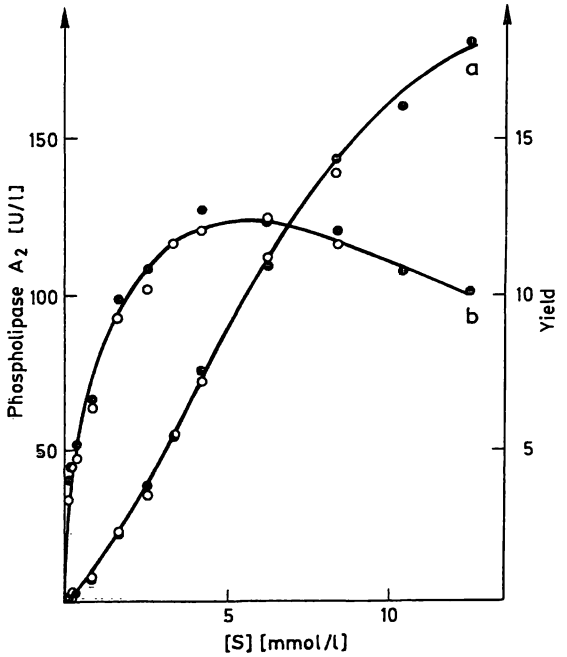


Fig. 3. Enzyme activity versus substrate concentration (a).
The yield of the labelled palmitic acid with respect to the labelled phosphatidylcholine
$\frac{\text{(counts/min (sample) − counts/min (blank)}}{\text{counts/min (total activity)}}$
plotted versus the phosphatidylcholine concentration (b).
The symbols represent the means of triplicates of two independent experiments.

The mean phospholipase $A_2$ activity of 30 serum samples of a healthy control group was 0.66 ± 0.11 U/l). A preliminary reference range up to 1.0 U/l was suggested. In a control group of 20 patients with gastrointestinal disease other than acute pancreatitis we found similar activities below 1 U/l. In serum samples of 50 patients with acute pancreatitis, catalytic activity ranged from 0.5 U/l up to 20 U/l.

As a typical clinical example, figure 4 shows a follow-up over 11 days of a 39 year-old male patient with severe acute pancreatitis. The patient underwent abdominal surgery because of extended pancreatic necrosis. Endotracheal intubation was carried out because of lung failure. Mechanical ventilatory support was given until day 9 after the onset of pain. During the following days he needed an additional oxygen supply. Serum samples were drawn every day and lung function was monitored by blood gas analysis. The pulmonary situation of the patient is described by the oxygenation index, which is the partial arterial oxygen pressure ($P_{aO_2}$) divided by the inspired fraction of oxygen ($F_{iO_2}$). A low oxygenation index indicates a bad lung function. It worsened twice during hospitalization at days 4 and 10. At the same times phospholipase $A_2$ activities showed two impressive peaks reaching about 10 U/l. In contrast, amylase and lipase values were only moderately increased in the beginning and normalized independently of the clinical course. They were not correlated to phospholipase $A_2$ activities.

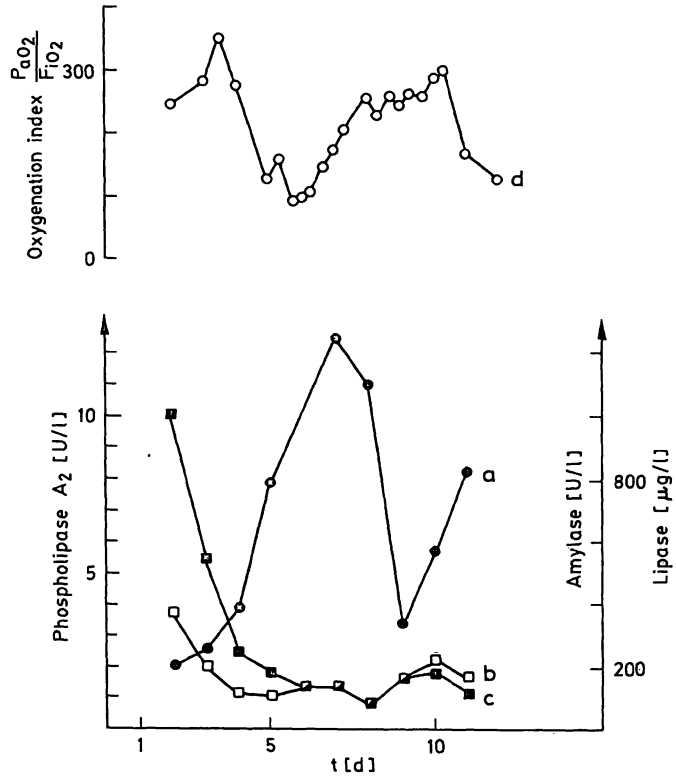


Fig. 4. Follow up of a sample patient with severe acute pancreatitis. Day 0 is the onset of the disease
a) phospholipase $A_2$-activity
b) pancreatic isoamylase activity
c) immunoreactive lipase
d) oxygenation index: partial arterial oxygen pressure divided by the inspired fraction of oxygen ($P_{aO_2}/F_{iO_2}$).

In intraoperatively drawn ascites samples of patients with acute pancreatitis (n = 6), elevated phospholipase $A_2$ activities between 1.6 U/l and 90 U/l were measured. Ascites and pleural effusions of patients with other diseases (n = 6) showed activities below 1 U/l.

## Discussion

The enzyme assay presented here is based on the method of *Shakir* (8), who measured phospholipase $A_2$ activities in post heparin plasma of rats and of purified snake venom enzyme. He did not detect any phospholipase $A_2$ activity in rat serum samples. To adapt this method for human serum, we had to modify the assay conditions.

Phospholipases $A_2$ of different origins need quite different reaction conditions for optimal catalytic activities (13). Using the original assay we found only minimal enzyme activities in human serum. The rather low substrate concentration of phosphatidylcholine (0.75 mmol/l) employed in the original assay was far from optimal range. Furthermore, the extremely high sample-reagent ratio of 2 : 1,3 led to high blank values and reduced activities. Preliminary experiments indicate that this effect was due to a high concentration of serum protein. Therefore, we used a reduced sample-reagent ratio of 1 : 5.

Also lipoprotein-bound phospholipids seem to be prone to a degradation by phospholipase $A_2$ (14). In normal sera the phospholipid concentration is in the range of 2 to 3 mmol/l, most of it (66 ± 9%) being phosphatidylcholine (15). In lipaemic sera this concentration can be increased several fold. In tracer assay systems, where only the added labelled phosphatidylcholine is measured, unlabelled serum phosphatidylcholine may greatly reduce the calculated enzyme activity, depending on the difference between the believed and the actual phosphatidylcholine concentration. This effect is minimized by the high substrate concentration and the high sample-reagent ratio in this assay.

Our aim was to minimize the sample volumes, without loss of test precision. Despite the low sample volume of 0.02 ml used in this assay, the measured signal was high enough to guarantee an inter- and intraassay precision, which fulfils the usual requirements for enzyme assays.

Phosphatidylcholine forms multilamellar liposomes in aqueous solutions, which hamper phospholipase $A_2$ activity. Therefore detergents like cholate have been introduced in the assay systems for phospholipase $A_2$ to form and stabilize monomeric micelles. The concentration of Na-deoxycholate (5 mmol/l) in this enzyme assay is relatively high and provides a substrate solution, which is stable over several days at room temperature. Therefore a rather long incubation period of 180 minutes could be used.

In enzyme assays using unlabelled substrates, the optimal reaction conditions are reached with substrate saturation and therefore maximal reaction velocity. Here the optimum was reached at a lower substrate concentration. Curve b in figure 3 shows the best yield of labelled palmitic acid. Other concentrations lead to a lowered yield and thus to diminished accuracy of the experiment. Measurements performed when the concentrations of substrate are too low will therefore be subject to high inaccuracy.

For easier handling an enzyme assay should be performed as a "one pot reaction". This was achieved by performing the incubation, extraction and washing of the upper phase in the same vial, without any loss in activity and without renewed contamination of the upper phase by phosphatidylcholine. Only liquid scintillation counting has to be performed in a separate scintillation vial. Using this simplified extraction procedure, we were able to measure 70 samples within the same series.

This method is strictly specific for the phospholipase $A_2$ activity. Phospholipase $A_1$ activity and phospholipase $A_1$-like activity, for example that of lipase, which can be elevated several fold in acute pancreatitis, do not influence this assay, because the fatty acids are selectively labelled in position 2.

The measurable catalytic activity concentration of phospholipase $A_2$ has been increased several fold compared to that achieved in the original method of *Shakir*. In patients with acute pancreatitis, it was increased up to 20 fold over the normal range, indicating a high sensitivity. In the patient reported in this paper, relatively low values for pancreatic isoamylase and lipase are not correlated with a severe course of acute pancreatitis, while elevated phospholipase $A_2$ values do correlate with poor lung function; thus phospholipase $A_2$ seems to behave differently from the other pancreatic enzymes.

The method described in this paper is easy to perform, has a good reproducibility and uses only commercially available reagents. It therefore qualifies as a tool for further studies on phospholipase $A_2$ and its role in acute pancreatitis.

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