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QUANTITATION OF SERUM PHOSPHOLIPASE A_2 BY ENZYME-DIFFUSION IN LECITHIN AGAR GELS

A COMPARATIVE STUDY IN MAN AND ANIMALS

By

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WESTERMARCK, E., L.-A. LINDBERG and M. SANDHOLM: *Quantitation of serum phospholipase A_2 by enzyme-diffusion in lecithin agar gels. A comparative study in man and animals.* Acta vet. scand. 1984, *25*, 229—241. — A sensitive gel-diffusion assay for determination of phospholipase A_2 was developed. PLA_2 standards, serum, faecal and pancreas homogenate samples with PLA_2-activity were allowed to diffuse from wells into agar-gels containing lecithin-membranes. The turbidity cleared radially upon PLA_2-activity. The diameters of the cleared zones showed a linear relationship with the log of the enzyme concentration. Serum samples resulted in some turbidity within the cleared zones. This interference originating from serum lipoproteins could be abolished by hydrophobic absorption. The gel-diffusion method was compared with two other methods for PLA_2, titrimetric and radiometric techniques. Analysis on 37 human patients with acute pancreatitis showed close interrelationship between these methods. The phospholipase A_2 activity in sera from man, the dog, the horse, the cow, the pig and the cat were almost equal, but much less than in the albino rat. No significant differences between PLA_2 activities in pancreatic samples were obtained in different animal species. Of the faecal samples, the cow had the lowest PLA_2 activity. Dogs suffering from pancreatic degenerative atrophy (PDA), had significantly reduced PLA_2 activity both in their pancreas and faeces but not in serum.

dog; cow; rat; cat; horse; blood; faecal test; pancreatitis; pancreatic insufficiency.

The problem of phospholipase determination is usually solved either by analyzing the lysophospholipid or free fatty acid produced. Most methods are based on fatty acid analysis by titrimetric (*Vogel & Zieve* 1960, *Zieve & Vogel* 1961), colorimetric (*Itaya* 1977) or radiometric procedures (*Warner & Benson* 1977, *Bosch*

& Aarsman 1979, *Tykkä et al.* 1984). Phospholipase substrates undergo physical changes upon hydrolysis. The splitting of micellar lecithins, such as yolk suspensions into the solubilizing lyso-compounds and fatty acids have been used as a basis for phospholipase A determination (*Habermann & Neumann* 1954, *Doizaki & Zieve* 1964). If the lecithin substrate is incorparated in agar-gels to produce turbidity, phospholipase A_2-activity can be seen as radially cleared zones at the periphery of the wells containing phospholipase A_2-activity (*Habermann & Hardt* 1972, *Moncla* 1979).

The determination of serum PLA_2-activity has been suggested as a specific method for diagnosing acute pancreatitis (*Nevalainen* 1980, *Schröder et al.* 1980, *Tykkä et al.* 1984).

The present report describes an enzyme-diffusion method for phospholipase A_2-activity based on agar gels containing lecithin membranes giving turbidity. The method was adapted especially for analysis of sera but also for pancreatic tissue and faecal samples. The method was compared with a titrimetric and radiometric method. The PLA_2 activities of man and different animals were examined.

MATERIAL AND METHODS

Phospholipase activities in serum, pancreatic tissue and faecal samples in man and different animals

PLA_2 activities of serum, pancreatic tissue and faecal samples were measured in 5 healthy individuals of the following species: horses, cows, pigs, cats, albino rats (Sprague Dawley commercial strain), and in 10 dogs. Also the samples from 8 dogs suffering from pancreatic degenerative atrophy (PDA) were measured. Thirty serum samples from healthy human blood donors and also 5 faecal samples were analyzed. Thirty-seven human serum samples from patients suffering from acute pancreatitis and 100 human serum samples obtained randomly from the daily material of the central laboratory of the Helsinki University Hospital were also analyzed.

Elaboration of phospholipase A_2 assay by the principle of enzyme diffusion in agar-gels containing lecithin membranes

By optimization for turbidity, good clearance by PLA_2 and sensitivity, the following gel composition and preparation method

was arrived at: Solution A: One gram of purified agar (Difco laboratories 0560-02) was dissolved in 50 ml 0.06 mol/l barbital buffer containing 0.003 mol/l Ca-lactate, pH 8.6. The agar was dissolved in a bath of boiling water and cooled to 56°C. Solution B: Another 50 ml barbital buffer included 200 mg phosphatidylcoline (Sigma P-3644, from soybeans), 800 mg BSA (Sigma A-8022) and 100 mg sodium azide as a preservative (dissolved at 56°C for 1 h). The mixture (B) was homogenized by Sorvall Omni-mixer for 2 min and sonicated for 15 s. Solution C contained 200 mg sodiumdeoxycholate in 10 ml distilled water at 37°C. Solutions A and B were pooled and magnetically stirred after which the solution C was added. The medium was poured without delay on petri dishes to form a 2 mm layer and allowed to solidify at room temperature. When ready, the plates were stored at + 4°C (stable up to 2 weeks).

Enzyme diffusion

Wells of 6 mm diameter were punched out of the gel and filled with PLA_2 standards. For preparation of the standard phospholipase dilutions, porcine pancreatic phospolipase (Sigma P-9139) was diluted 2×10^{-6} by distilled water containing

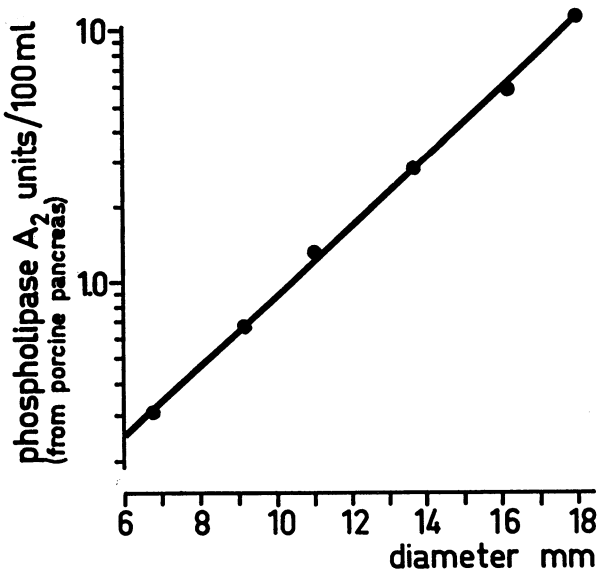

Figure 1. Relationship between the diameters of cleared zones with the concentration of PLA_2 (from porcine pancreas).

1 % BSA. The standard dilution series was prepared further in double dilution steps. The samples were allowed to diffuse out of the wells for 24 h at 37°C in a humid chamber. A double dilution series of a serum with high PLA_2 was run parallelly. The standard curves from PLA_2 and serum dilutions were plotted on a semilogarithmic scale upon which a straight line was obtained from diameters of the cleared zones versus logarithms of the amounts of PLA_2 (Fig. 1).

Analysis of plates by transmission electron microscopy (turbid plates/cleared zones)

Samples of gels (Ø 0.1 mm) were fixed in phosphate buffered 3 % glutaraldehyde, pH 7.2, for 1 h at 20°C. After overnight rinsing in cold phosphate buffer, post-fixation was performed in 1 % osmium tetroxide for 1 h at 20°C. The samples were then dehydrated and embedded in Epon. Thin sections were contrasted with lead citrate and uranyl acetate. The sections were examined in a JEOL JEM-100 S transmission electron microscope.

Visualization of PLA_2-activity in electropherogrammes

A serum sample containing PLA_2-activity was separated by electrophoresis in 1 mm thick agar gel (1 % Agarose A 37 prepared in the Barbital-Ca-lactate buffer, pH 8.6). The electrophoresis was run at 100 V/10 cm for 90 min at + 8°C. After electrophoresis, a 1 mm layer of the phospholipid agar was poured on the top of the electrophoresis layer and allowed to solidify. The plate was left to diffuse for 24 h.

To see whether the PLA_2 activity was associated with immunoglobulins, a serum sample with high PLA_2 activity and serial dilutions of rabbit anti-IgG, -IgM and -IgA (Boehringwerke) were all allowed to diffuse against each other from wells 1 cm apart into the phospholipid agar (= combined immuno- and enzyme diffusion). The location of the immunoprecipitates and the boundaries of the cleared zones were compared.

Problem of turbidity from interfering factors in sera

As all the serum samples gave a turbid zone around the wells (inside the cleared zone), attemps were made to analyze which serum factors interferred and how this interference could be eliminated. As the turbidity seemed to originate from lipoprotein

interaction, the following methods known to eliminate lipoproteins were tested: Aether extraction, precipitation by 12 % polyethylene-glycol-6000, 6.8 % caprylic acid, 5 % Mg Cl_2, 5 % dextrane and hydrophobic absorption by phenyl-or octyl sepharose. As hydrophobic absorption showed to be the most effective method it was selected as the method for abolishing the interfering factors during further studies. The serum samples were mixed with an equal volume of packed octyl sepharose (Pharmacia Fine Chemicals), which had been washed by distilled water and incubated for 30 min at room temperature and then spun at 10,000 g/2 min by an Eppendorf centrifuge. After this treatment the supernate gave a completely clear zone of PLA_2 activity (Fig. 2).

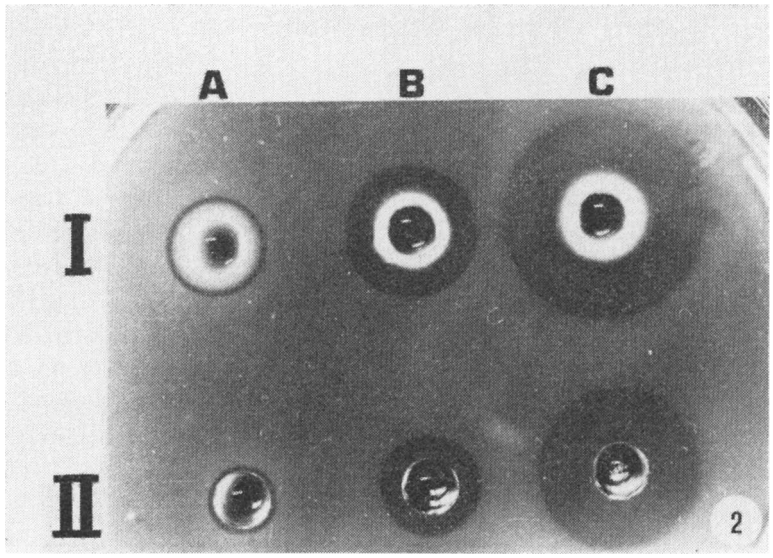

Figure 2. Enzyme diffusion of 3 sera in agar-gels containing phosholipid membranes, diffusion time 24 h. Serum A is a control serum, B and C are sera from patients with acute pancreatitis. I upper panel: untreated sera. II lower panel: the sera were absorbed by hydrophobic beads (octyl sepharose) before transferring into the wells. This absorption removed the turbidity originating from interaction of serum lipoproteins with the medium.

To confirm that phospholipase C and D (Sigma PLC P-7633, Sigma PLD P-7758) did not affect the assay, they were included in separate wells as well as mixed together with PLA_2.

Correlation of the enzyme-diffusion assay with existing methods

Serum samples of 37 human patients with acute pancreatitis were analyzed parallelly with the present method, a titrimetric method (*Vogel & Zieve* 1960) and a radiometric assay (*Tykkä et al.* in press).

The within-assay precision was determined from 37 duplicate measurements on the same plate. The between assay precision was determined on 3 serum samples showing low, medium and high PLA_2 activities by pipetting the samples on 30 different plates all containing the dilution series of PLA_2 standards. The effect of storage of the sera was analyzed by storing all the samples at + 4 and —20°C for 2 weeks after which they were reanalyzed.

The determination of PLA_2 from the faecal and pancreatic samples

The faecal samples were diluted 1 : 10 with PBS (phosphate buffered saline: NaCl 8.0 g, KCl 0.2 g, $Na_2HPO_4 \times 2H_2O$ 0.25 g, KH_2PO_4 0.2 g, $CaCl_2$ 0.1 g, $MgCl_2 \times 6H_2O$ 0.1 g, H_2O ad 1 l) and homogenised by an Ultra-Turrax homogeniser (Janke & Kunkel KG).

Fresh pancreas was diluted 1/100 with PBS and homogenised by a Sorvall Omni-Mixer for 1 min cooled with ice. The PLA_2 precursor of the pancreas homogenates was activated with trypsin (Trypure® Novo Industries, Denmark). Trypsin (0.005 mg/ml PSB) was mixed with an equal volume of the homogenate. Before pipetting the faecal and pancreatic samples on the plate they were mixed with octyl sepharose as the serum samples.

RESULTS

The lecithin was embedded within the gels in the form of phospholipid membranes (Fig. 3). These structures became completely dissolved upon PLA_2-activity originating either from pure pancreatic PLA_2 or serum activity. When examined with electron microscopy no membrane structures were left within the digested zones. Other types of phospholipases did not affect the assay by clearing. Phospholipase C gave a turbid zone around the well and phospholipase D was without effect. There were two ways of circumventing the disturbing effect from lipoproteins. The areas of the turbid zones could be excluded mathematically

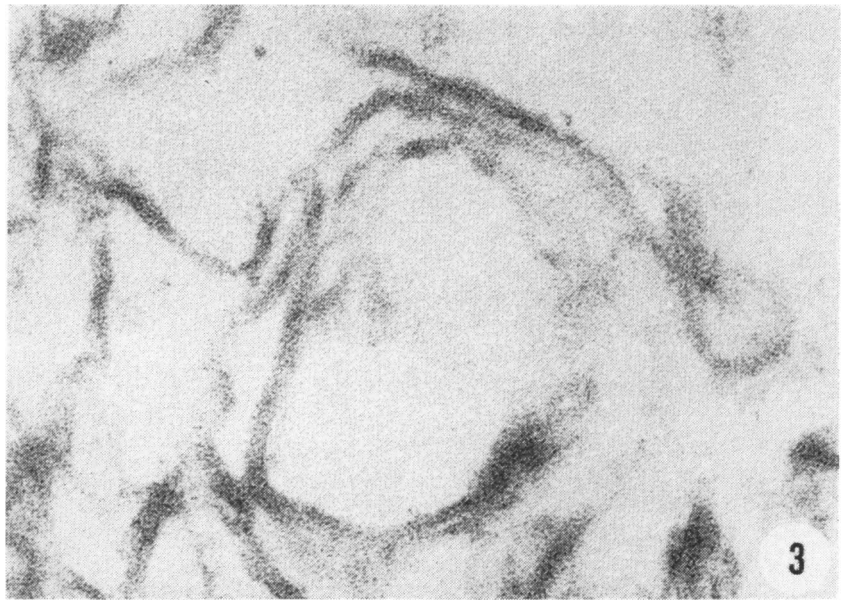

Figure 3. Analysis of opalescent plates by electron microscopy. Note that the phospholipid is organized as membrane structures within the agar gel. When cleared zones were analyzed, the structures had completely disappeared ($\times$ 180,000).

by subtraction. When the area of the turbid zone was subtracted from the total area of the cleared zones (as calculated from the diameters) the difference showed better correlation with the PLA_2-activities as determined by the two other assays than the whole zones. Alternately the interfering turbidity could be removed by hydrophobic absorption with octyl sepharose, indicating that the interference comes from hydrophobic proteins in serum.

The phospholipase activity of serum showed the same electrophoretic mobility as γ-globulin, but was not associated with immunoglobulins as indicated by independent diffusion.

The PLA_2-activities obtained by the present enzyme diffusion technique showed a relatively good correlation with the activities obtained by the two other techniques (titrimetric and radiometric techniques). The correlation coefficient (r) between the enzyme diffusion technique with both other methods was 0.87 (Figs. 4, 5). This was of the same order as the interrelationship between the titrimetric assay and radiometric assay (Fig. 6). The smallest

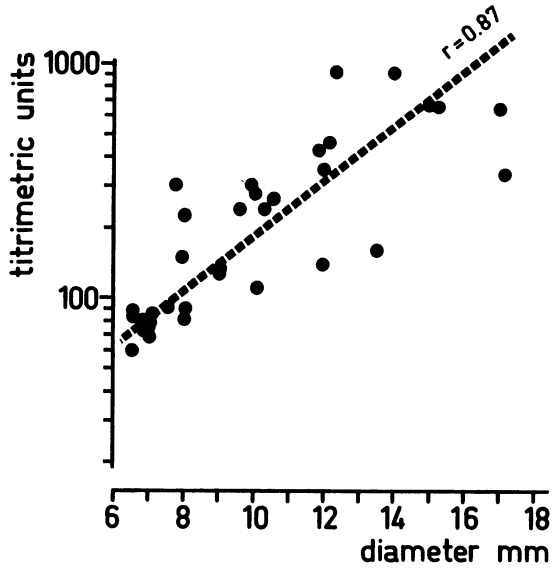

Figure 4. Correlation of PLA_2-activities as determined on enzyme diffusion on agar gels containing lecithin membranes with a titrimetric procedure for PLA_2. The serum material consisted of 37 sera from patients with acute pancreatitis.

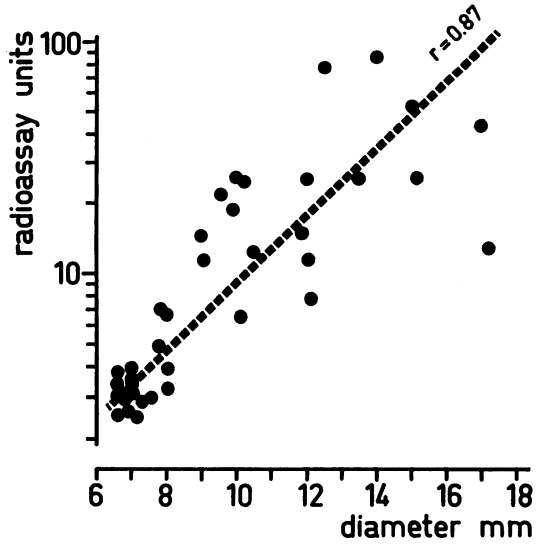

Figure 5. Correlation of PLA_2-activities as determined on enzyme diffusion on agar gels containing lecithin membranes with a radiometric procedure for PLA_2. The serum material consisted of 37 sera from patients with acute pancreatitis.

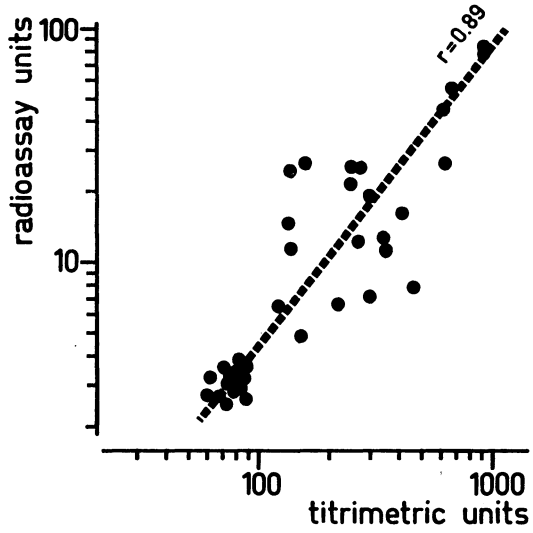

Figure 6. Relationship between the PLA_2-values obtained from the titrimetric and radiometric procedures.

amount of pancreatic PLA_2-activity which could be distinguished from zero is obtained when the cleared zone is 0.5 mm wider (Ø 6.5 mm) than the well (Ø 6.0 mm). This means that the lowest detectable concentration using porcine pancreatic phospholipase is 0.3 units/100 ml (Sigma units).

PLA_2-activities of sera from healthy human beings and the studied animal species except the albino rat, fell in the range of 6.5—8.5 mm diameter which means that the sensitivity of the assay is good enough for screening sera for PLA_2-activity. The scatter of duplicate measurements (± s) in the agar diffusion technique was 6 % from the mean activities (coefficient of variation) increasing to 9 % when inter-plate and day-to-day variations were included. Upon storage of serum samples (either at + 4°C or —20°C) some activity was lost (0—20 %) and the loss seemed to be inconsistant from sample to sample. Heat treatment of serum (56°C/30 min) was not seen to affect the result. Strongly hemolyzed blood samples seemed to give "false" positive results and should be evaluated with care. The increase of serum PLA_2-activity in man is clearly associated with acute pancreatitis. Of the 37 serum samples originating from patients with acute pancreatitis, 19 showed an increased PLA_2-activity. However, increased PLA_2-levels were not completely pathognomonic

Table 1. Normal phospholipase A_2 activities in serum, pancreas homogenate and faecal samples in man and different animals as given in diameters of the cleared zones/mm ± s on agar-gel containing a substrate. The diameter of the well is 6 mm.

	Man	Dog (normal)	PDA dog	Horse	Cow	Pig	Cat	Rat
Serum	7.0 ± 0.5 (n = 30)	7.2 ± 0.6 (n = 10)	7.0± 0.3 (n = 8)	6.7 ± 0.2 (n = 5)	6.7 ± 0.1 (n = 5)	6.8±0.2 (n = 5)	6.7±0.1 (n =5)	14.5±0.4 (n = 5)
Pancreas	—	22.2 ± 1.4 (n = 10)	7.2 ± 1.8 (n = 8)	24.7 ± 2.6 (n = 5)	24.1 ± 0.5 (n = 5)	23.7±0.9 (n = 5)	19.4±1.1 (n =5)	19.3±0.6 (n = 5)
Faeces	22.3 ± 1.9 (n = 5)	18.1 ± 1.4 (n = 10)	7.4 ± 1.8 (n = 8)	17.1 ± 1.5 (n = 5)	10.7 ± 0.6 (n = 5)	19.6±1.1 (n = 5)	19.9±2.1 (n =5)	19.9±0.7 (n = 5)

The phospholipase activity in Sigma units (y) can be obtained from the diameter of the cleared zone (x) by using the formula $y = ae^{bx}$ where a = 0.04 and b = 0.31.

PDA = pancreatic degenerative athrophy.

for pancreatitis. Among the 100 hospitalized patients none of which had acute pancreatitis, 92 were within the normal PLA_2-range (< 0.45 units), 7 had slightly increased levels (0.45—0.7 units) and 1 patient with terminal pneumonia had 2.1 units/100 ml.

The PLA_2 activities in sera, pancreas and faecal samples in different animal species and man are presented in Table 1. The PLA_2 activities in the serum samples of the different animals were of the same magnitude except the albino rat, which had a serum PLA_2 level about 5 times higher than the other tested species. The faecal and pancreatic samples gave sometimes some turbidity inside the cleared zone which could be abolished with absorption by octyl sepharose. The PLA_2 activity in the pancreas samples did not vary significantly between the species examined. The cow had significantly lower faecal PLA_2 activity than the other animal species and man. The dogs with pancreatic degenerative atrophy (PDA) had only traces of PLA_2 activities both in pancreas and faecal samples, but the serum PLA_2 activity was equal to normal dogs.

DISCUSSION

The advantages of the radial enzyme diffusion test for PLA_2 seems to be as follows: 1. The phospholipase reaction is visible and the technique can be combined with other techniques such

as immunodiffusion and electrophoresis. 2. A large number of samples can be applied and analyzed simultaneously. 3. The quantitation is easy and cheap. Using a semilogarithmic plot, one can interpolate the phospholipase activities from the standards. 4. The sensitivity is good. The principal disadvantage of the agar-diffusion method as compared with other available methods it the long incubation time (24 h at 37°C). The plates can be incubated at room temperature as well, but the sensitivity decreases slightly. The gel-diffusion principle for determination of phospholipase activities has been suggested earlier (*Habermann & Hardt* 1972, *Moncla* 1979). However, these methods did not show sensitivities good enough to analyze serum samples. The present method differs from these earlier techniques in a few key points, the phospholipid (lecithin) is present as membrane structures (Fig. 3). By including sodiumdeoxycholate, the sensitivity was markedly increased. The preparation is critical in order to obtain stable plates with maximum turbidity. The given procedure should be followed accurately. The correlation with the two other methods for PLA_2 might raise some doubts about the accuracy of the method (Figs. 4, 5). Apparently the scatter seems to originate from the effect of storage on PLA_2 activity. The correlation between different techniques suffered from the fact that the analyses were not all performed on the same date. Fresh samples should be used whenever possible. Heavily hemolyzed samples showed phospholipase activity and hence such samples should be evaluated with care.

Increased serum phospholipase activity seemed to be relatively specific for acute pancreatitis. Hower, some phospholipase activity was found in non-pancreatic disases as well. Serum phospholipase in pancreatitis patients seemed to be included within the electrophoretic gammaglobulin fraction, which findings is in agreement with earlier reports (*Scherer et al.* 1976). Further research is required to see whether pancreas derived and non-pancreatic phospholipases could be differentiated.

The radial diffusion test for PLA_2 proved to suit for faecal and pancreas homogenate samples. The PLA_2 activities in serum, faecal and pancreas homogenate samples were generally on an equal level in man and the different animal species. The only exception was the high serum PLA_2 contents in the albino rat, this is in agreement with an earlier report by *Sing et al.* (1979). Although the pancreatic and faecal PLA_2 activities of PDA dogs

were very much lower than in normal dogs, there were no significant differences in serum PLA_2 activities. This might indicate that normal serum PLA_2 activity is not pancreatic in origin.

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REFERENCES

van den Bosch, H. & A. J. Aarsman: A review of methods of phospholipase A determination. Agents Actions 1979, *9*, 382—389.

Doizaki, W. M. & L. Zieve: Turbidimetric assay for phospholipase A. J. Lab. clin. Med. 1964, *63*, 524—536.

Habermann, E. & K. L. Hardt: A sensitive and specific plate test for the quantitation of phospholipases. Analyt. Biochem. 1972, *50*, 163—173.

Habermann, E. & W. Neumann: Die Hemmung der Hitzekoagulation von Eigelb durch Bienengift — ein Phospholipase-Effekt. (To prevent hot coagulation of egg yolk with venom — a phospholipase effect). Hoppe - Seylers Z. physiol. Chem. 1954, *18*, 179—193.

Itaya, K.: A more sensitive and stable colorimetric determination of free fatty acids in blood. J. Lipid. Res. 1977, *18*, 663—665.

Moncla, B. J.: Method of testing for phospholipases using a composition containing a uniform dispersion of a phospholipid. U.S. Patent 1979, Feb. 20, no. 4, *140*, 579.

Nevalainen, T. J.: Review: The role of phospholipase A in acute pancreatitis. Scand. J. Gastroent. 1980, *15*, 641—650.

Scherer, R., W. Huber-Friedberg, A. Salem & G. Rauhenstroth-Bauer: Phospholipase A_2 activity in human γ-globulin fraction. Hoppe-Seylers Z. physiol. Chem. 1976, *357*, 897—902.

Schröder, T., E. Kivilaakso, P. K. Kinnunen & M. Lempinen: Serum phospholipase A_2 in human acute pancreatitis. Scand. J. Gastroent. 1980, *15*, 633—636.

Singh, C., V. Ramesh & K. Anjaneyulu: A comparative study of serum phospholipase A_2 in man & animals — some biochemical & physiological implications. Indian. J. exp. Biol. 1979, *17*, 50—57.

Tykkkä, H. T., K. L. Mahlberg, E. J. Vaittinen, J. E. Railo, P. J. Pantzar, S. Sarna & T. Tallberg: Serum phospholipase A_2 activity in human acute pancreatitis. Scand. J. Gastroent. 1984(in press).

Warner, T. G. & A. A. Benson: An improved method for the preparation of unsaturated phosphatidylcholines: acylation of sn-gly-

cero-3-phosphorylcholine in the presence of sodium mehylsulfinylmethide J. Lipid. Res. 1977, *18*, 548—552.

Vogel, W. C. & L. Zieve: A lecithinase A in duodenal contents of man. J. clin. Invest. 1960, *39*, 1295—1301.

Zieve, L. & W. C. Vogel: Measurement of lecithinase A in serum and other body fluids. J. Lab. clin. Med. 1961, *57*, 586—599.

SAMMANFATTNING

Kvantitering av serum fosfolipas A_2 genom enzymdiffusion i lecitin agar-gel.

En känslig gel-diffusionsmetod för bestämning av fosfolipas A_2 (PLA_2) utvecklades. PLA_2 standards, serum, feces- och pancreashomogenat med PLA_2-aktivitet fick diffundera från fördjupningar i agargeler innehållande lecitinmembraner. Som en följd av PLA_2-aktivitet skedde en cirkulär uppklaring av gelen. Diametrarna av de uppklarade zonerna stod i lineärt förhållande til logaritmen av enzymkoncentrationen. Serumprov resulterade i en vis grumlighet inom de uppklarade zonerna. Denna störning, som antagligen beror på lipoproteiner i serum, kunde avhjälpes med hydrofobisk absorption. Geldiffusionsmetoden jämfördes med två andra metoder för PLA_2, en titrimetrisk och en radiometrisk teknik. Analys av 37 humanpatienter med akut pancreatit visade en nära samstämmighet med dessa metoder. Fosfolipas A_2-aktiviteten i sera från människa, hund, häst, nöt, svin och katt var nästan lika, men mycket lägre än hos albinoråtta. Inga signifikanta skillnader mellan PLA_2-aktiviteterna i pancreasprov från olika djurslag kunde konstateras. Bland de fecala provena hade nöt den lägsta PLA_2-aktiviteten. Hundar med degenerativ pancreasatrofi (PDA), hade signifikant sänkt PLA_2-aktivitet både i pancreas och feces, men inte i serum.

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