Simple assay for glycerophospholipid hydrolase activity of postheparin plasma

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Summary An assay using radioactive substrates is described that permits rapid determinations of glycerophospholipid-hydrolyzing activity in postheparin plasma or its fractions. Optimal conditions are described for hydrolysis of phosphatidylcholine and phosphatidylethanolamine. A minimum of only 2 μl of normal postheparin plasma is used and no extraction of the reaction products is required before their separation by thin-layer chromatography. We found that after an optimal heparin dose of 60 units/kg body weight the rate of hydrolysis for diacyl glycerophosphocholine and diacyl glycerophosphoethanolamine is 1.16 μmoles/ml per hr and 22.4 μmoles/ml per hr, respectively.

Supplementary key words ^{14}C-labeled phospholipids · deoxycholate · thin-layer chromatography

Enzymatic activity that catalyzes the hydrolysis of acyl esters and acyl thioesters appears in the blood of mammals after intravenous administration of heparin (1, 2). Two types of hydrolytic enzymes have been isolated from postheparin plasma of both animals (3) and man (4). One of the enzymes has properties similar to those of an enzyme released from liver (4, 5). This enzyme catalyzes the hydrolysis of glycerophospholipids (6–8), triglyceride (7, 9, 10), monoglycerides (9) and thioacylesters (11). The other enzyme has properties similar to those of enzymes released from extrahepatic tissues (4) and preferentially catalyzes the hydrolysis of triglycerides (8, 12).

The need for a rapid and specific assay to measure the hydrolysis of glycerophospholipids catalyzed by these enzymes stimulated development of a new approach. The assay is derived from the work of Vogel and Bierman (13) which describes conditions for the exclusive hydrolysis of fatty acid from the 1-position of glycerophospholipids. Because detection of radiolabeled fatty acid products could be made more sensitive than titration or determination of phosphorus in the monoacyl product, a glycerophospholipid with a radioactive fatty acid esterified to the 1-position of glycerol was chosen as substrate for this assay.

Abbreviations: GPE, *sn*-glycero-3-phosphoethanolamine; GPC, *sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography; DOC, deoxycholate.

This approach permits simple and rapid serial sampling of glycerophospholipid hydrolyzing activity in postheparin plasma or its fractions, and either 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (GPE) or 1,2-diacyl-*sn*-glycero-3-phosphocholine (GPC) can be used as substrates. The unique features are that only 2–20 μl of postheparin plasma are used and no extraction of the reaction products is required before their separation by thin-layer chromatography (TLC). This report describes a procedure for synthesis of the radioactive substrates, data on the assay, and biological reproducibility and optimal heparin dosage.

Materials

[1-^{14}C]Palmitic acid (99% pure) was purchased from Applied Science Laboratories, State College, PA. Egg yolk and phospholipase D were obtained from Grand Island Biological Company, Grand Island, NY. Ethanolamine was obtained from Eastman Organic Chemical, Rochester, NY. The detergents Triton X-100, deoxycholate, and cetyltrimethylammonium-bromide were obtained from Sigma Chemical Company, St. Louis, MO. Silica gel CC4 100–200 mesh was obtained from Mallinckrodt, St. Louis, MO. LQD and Q6 TLC plates were obtained from Quantum Industries, Fairfield, NJ. *Crotalus atrox* venom was obtained from Ross Allen, Silver Springs, FL. Sodium heparin was obtained from Organon. Inc., West Orange, NJ. All other substances used were reagent grade.

Procedures

Substrates: preparation of diacyl-GPC and diacyl-GPE. Diacyl-GPC was purified from egg yolk by column chromatography using procedures described by Singleton et al. (14). Diacyl-GPE was transphosphatidylated from diacyl-GPC in a 70% yield using cabbage phospholipase D and ethanolamine, essentially as described by Yang, Freer, and Benson (15) and modified by Jezyk and Hughes (16). The purified diacyl-GPC and diacyl-GPE were observed as single spots on Quantum Q6 TLC plates developed in the following solvents: neutral, chloroform–methanol–water 65:25:4; acidic, chloroform–methanol–acetic acid–water 50:25:8:3; basic, chloroform–methanol–ammonia 65:25:4. A sequential detection system using ninhydrin, molybdate (17), and sulfuric acid (charring) was used with each solvent system. The absence of phosphatidic acid in the diacyl-GPE product was assured by the presence of only one spot when the TLC plate was developed in the basic solvent system.

Substrates: preparation of radioactive diacyl-GPC and diacyl-GPE. The method of Robertson and Lands (18) was used to prepare 1-[(1-^{14}C)palmitoyl]-2-acyl-GPC

(sp act 15.4 μCi/μmole). A 60% yield with respect to radioactivity was obtained in the synthesis from 2-acyl-GPC (19) and [1-^{14}C]palmitic acid (sp act 55.7 μCi/μmole). 1-[(1-^{14}C)Palmitoyl]-2-acyl-GPE was prepared in a 70% yield from 1-[(1-^{14}C)palmitoyl]-2-acyl-GPC by transphosphatidylation as described above (16). Enzymatic hydrolysis of the molecule with snake venom (20) and isolation of the products by TLC revealed that 95% of the radioactivity in the glycerophospholipid was esterified to the 1-position of glycerol. The distribution of radioactivity after hydrolysis by snake venom was as follows: diacyl phosphoglyceride, 0.3%; fatty acid, 4.8%; and 1-[(1-^{14}C)acyl]phosphoglyceride, 94%. The radioactive glycerophospholipids obtained were 99.8% radiochemically pure as determined by TLC on silica gel. All lipids were stored at −20°C in hexane and the stability was checked periodically by TLC on silica gel using the solvent systems described above.

Assays: diacyl-GPE substrate. In the routine batch preparation of the diacyl-GPE substrate, labeled and unlabeled glycerophospholipids were mixed to give 6.75 μmoles of 1-[(1-^{14}C)palmitoyl]-2-acyl-GPE with a specific activity of 0.21 μCi/μmole. After removal of organic solvent with nitrogen, the lipid was mixed (with a Vortex mixer) for 5 min at room temperature with 0.25 ml of 0.14 M glycine, pH 9.9, containing 27 mM deoxycholate (DOC). The resulting solution was sufficient for the assay of nine samples plus an aliquot to determine the total radioactivity in the assay. Multiple volumes of this solution were prepared daily depending upon the anticipated requirements.

To each assay vial was added 25 μl of substrate mixture (0.675 μmole of diacyl-GPE). Enzyme preparation and/or water was then added to obtain the final assay volume of 45 μl. The vial was gently shaken, capped, and incubated at 37°C for exactly 15 min. The reaction was interrupted by placing the vial in an ice bath. Separation of reaction products was initiated by applying two 20 μl aliquots of one incubation solution to one channel of a prescored 5 × 20 cm Quantum, LQD TLC plate to which carrier oleic acid had previously been applied. After four incubation mixtures were applied to one plate, it was immediately developed for about 2 min with acidic solvent. The chromatography plate was dried for 1 hr at room temperature and then developed for 40 min in petroleum ether–diethyl ether–acetic acid 72:21:1. After the plate was sprayed with 0.25% dichlorofluorescein in ethanol, the lipids were located by ultraviolet light. The area containing fatty acid was scraped into scintillation vials and counted in a liquid scintillation counter (21).

Diacyl-GPC substrate assay. In the routine batch preparation of the diacyl-GPC substrate, labeled and unlabeled glycerophospholipid were mixed to give 4.5 μmoles of 1-[(1-^{14}C)palmitoyl]-2-acyl-GPC with a specific activity of 1.75 μCi per μmole. After removal of solvents, the lipid was mixed (with a Vortex mixer) for 5 min at room temperature with 0.25 ml of 0.14 M glycine, pH 9.9, containing 18 mM DOC. The resulting solution was sufficient for the assay of nine samples plus an aliquot to determine the total radioactivity in the assay. This reaction mixture was processed exactly as described for the diacyl-GPE incubation system, except the time of incubation was 60 min.

Nonradioactive assay. The assay as described by Vogel and Bierman (13) was used without modification.

Preparation of subjects. Healthy male volunteers, 18 to 56 years of age, consuming a typical American diet and with normal plasma lipids (cholesterol <250 mg/dl, triglyceride <150 mg/dl), were studied 12 hr postprandially with only water being permitted after the evening meal. Unless otherwise indicated, postheparin plasma was obtained after injection of 60 units heparin/kg body wt into an unoccluded vein. After a standard interval of 10 min, or varying intervals in some studies, blood was obtained from an unoccluded vein in the opposite arm using a dry syringe. The blood was placed in a centrifuge tube containing 100 units of heparin and inverted. The blood was handled thereafter at 0–5°C. Plasma was removed after slow centrifugation and either assayed immediately or aliquots were stored at −15°C for periods up to 6 months.

Results and discussion

The anionic detergent DOC was used in the routine assay after investigation showed it to be more effective than the nonionic detergent, Triton X-100. Triton X-100 can replace DOC in the assay; however, the enzyme rate at the optimal concentration of Triton X-100 is about one-half the rate for the optimal concentration of DOC. The cationic detergent, cetyltrimethylammoniumbromide, catalyzed a rapid chemical hydrolysis of the glycerophospholipids, and therefore was not suitable to evaluate the enzymatic hydrolysis.

Fig. 1 presents the effects of variation of some assay conditions. As shown in Fig. 1A, substrate was not rate limiting at 10 mM diacyl-GPC and 15 mM diacyl-GPE, the concentrations used in the routine assay. The optimal molar ratio of the anionic detergent (DOC) to substrate has been found to be 0.9–1.1 (Fig. 1B). Within this range, values for enzymatic activity differed less than 10%, but at lower or higher ratios, there were rapid decreases of

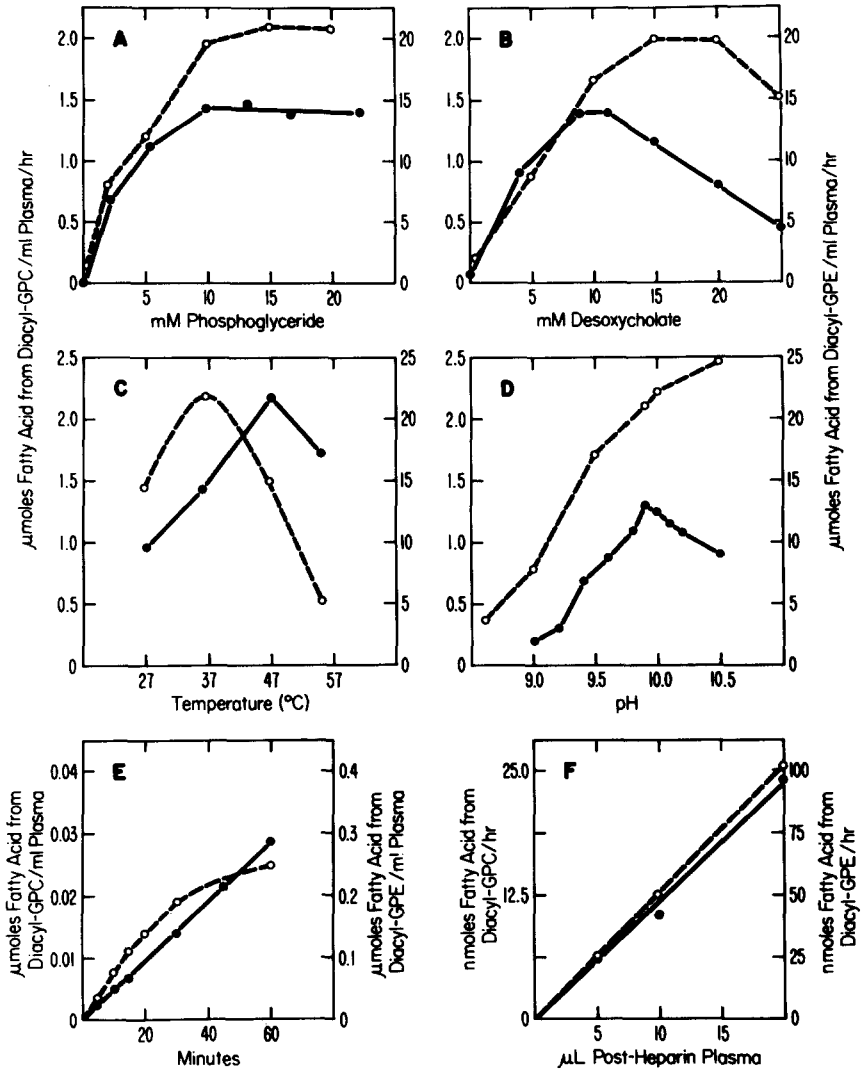

Fig. 1. Relation of enzyme activity measured under standard conditions to (A) substrate concentration; (B) deoxycholate concentration; (C) assay temperature; (D) pH of assay; (E) incubation time; and (F) enzyme concentration of postheparin plasma. (● —— ●) Diacyl-GPC and (○ - - - ○) diacyl-GPE.

rates of hydrolysis. The optimal ratio of nonionic detergent Triton X-100 to substrate was found to be 0.1, but at lower or higher ratios, the hydrolysis rate decreased rapidly.

In the routine assay at 37°C, hydrolysis was about 22 times greater for diacyl-GPE than for diacyl-GPC (Fig. 1C). At 47°C the rate of enzymatic hydrolysis for diacyl-GPE decreased but the rate for diacyl-GPC increased. Apparently, the diacyl–GPC/DOC micelle protected the enzyme from thermal denaturation.

Enzymatic activity plotted against pH of incubation showed an increase in enzyme activity from pH 9.0 to 9.9 for both glycerophospholipid substrates (Fig. 1D). Above pH 9.9 the rate of hydrolysis decreased for diacyl-GPC but continued to increase for diacyl-GPE. A pH level of 9.9 was chosen for the routine assay because above this pH chemical hydrolysis gave elevated blanks. The blank at pH 9.9 represented 0.03 µmoles and 0.6 µmoles of fatty acid from diacyl-GPC and diacyl-GPE, respectively. Liberation of fatty acid equal to twice the blank was accepted as the lower limit of enzyme activity. The minimum volume of normal postheparin plasma needed to detect enzymatic hydrolysis was approximately 2 µl. The variation for duplicate sample analysis was less than 5%.

Enzyme activity was linear for 15 min with diacyl-GPE and for at least 60 min with diacyl-GPC (Fig. 1E). The rate of hydrolysis for both substrates was linear with different amounts of postheparin plasma (Fig. 1F). An increase of ionic strength in the incubation mixture (up to 1.0 M NaCl) did not alter the

TABLE 1. Effect of monoglyceride on postheparin plasma glycerophospholipid hydrolysis rate

Added Lipid	Product	
Monoglyceride	FFA % of Control	Diglyceride % of FFA
mM		
0	100	5.0
0.1	100	7.8
0.5	100	14.5
1.0	105	8.0
5.0	63	13.0
10.0	46	16.7

Phosphoglyceride substrate was assayed in the presence of the indicated amount of monoglyceride. Free fatty acid was determined as described for the standard assay. Diglyceride was separated by TLC using the solvent petroleum ether–diethyl ether–acetic acid 73:23:1. Radioactivity was determined as described for the standard assay.

enzymatic activity. Calcium chloride at 0.05 mM inhibited the reaction rate by 60%.

Vogel et al. (22) first demonstrated that the enzyme from postheaprin plasma could transacylate fatty acid from the glycerophospholipid substrate to various water soluble alcohols. Recently Waite and Sisson (23) have shown that an enzyme from liver plasma membrane, assayed in the absence of detergent, preferentially transferred fatty acid from glycerophospholipid to monoglyceride and also hydrolyzed monoglyceride. Since postheparin plasma from normal and hyperlipidemic patients would contain various concentrations of monglyceride, we tested the utilization of monooleoylglycerol as an acyl acceptor. Various concentrations of the acceptor monoglyceride, in the presence of a fixed concentration of substrate, produced a maximum transacylation of only 16.7% of the substrate hydrolysis rate (**Table 1**). Our results indicate that disruption of the liposome by the detergent increased the availability of water to the enzyme and shifted the system from a transferase reaction toward the production of free fatty acid. Since the physiologic concentration of monoglyceride is well below 60 mg/dl (1 mM in the assay), interference by monoglyceride in our assay would be negligible. Mixing preheparin plasma or chylomicrons from a Type I hyperlipidemia patient with postheparin plasma from a normal subject did not alter the enzymatic activity in normal postheparin plasma.

Reliability and validity. Rates of diacyl-GPC hydrolysis using our assay were comparable to the nonradioactive assay described by Vogel and Bierman (13). In seven healthy subjects the mean activity using the nonradioactive assay was 1.20 μmoles/ml per hr compared to a mean of 1.16 μmoles/ml per hr for the radioactive assay; this difference was insignificant. The rates of diacyl-GPE hydrolysis in postheparin plasma from normal subjects was 22.4 μmoles/ml per hr using our assay. This is similar to results reported by Vogel, Brunzell, and Bierman (24), who found 20 μmoles/ml per hr. Thus, the radioactive and nonradioactive assays are in good general agreement.

The reliabilty of the assay was determined by retesting the same subjects at biweekly intervals for 10 months, using the same heparin dose. Retest of the same individual yielded differences from the mean of less than 10% in 16 of 22 assays and less than 15% in 19 of 22 assays. Seven normal male subjects were restudied 1 week after the initial test. Test–retest reliability afforded a mean difference between the two values of 7% with a range of 1–13%. No change in activity was found with storage of frozen postheparin plasma for periods up to 6 months so long as the sample remained frozen.

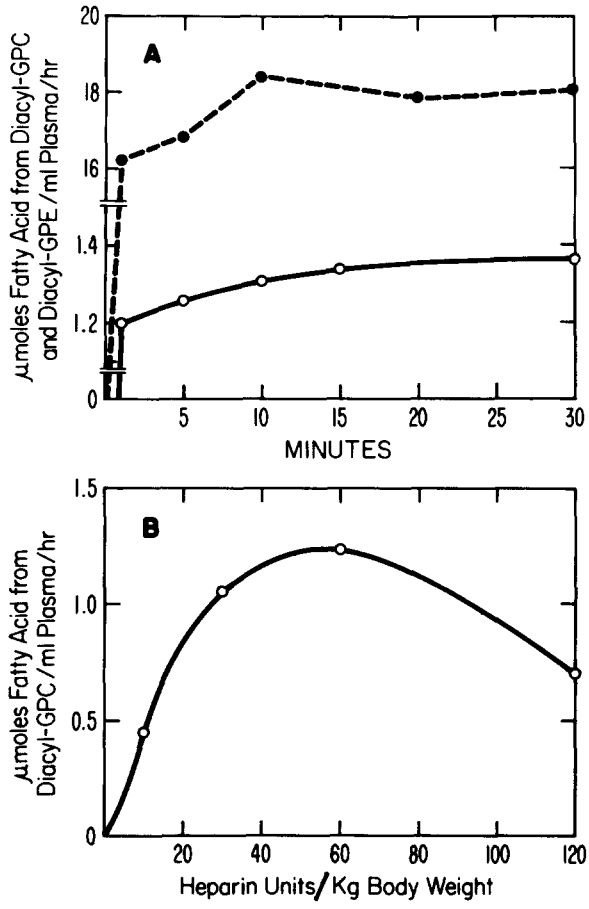

Fig. 2. (A) Temporal relationships in release of glycerophospholipid hydrolyase activity. Sixty units heparin/kg body wt was administered to normal human subjects. (○ —— ○) Diacyl-GPC and (● – – – ●) diacyl-GPE. (B) Effect of heparin on plasma concentration of glycerophospholipid hydrolyase activity. Heparin was administered as described in the text.

Heparin response. The optimal heparin dose and the timing of blood collection for males were investigated. After a standard dose of 60 units of heparin/kg body wt was administered, blood was obtained after 1, 5, 10, 15, and 30 min (**Fig. 2A**). Glycerophospholipid hydrolysis was 80% of maximum within 1 min and 90% of maximum at 5 min. By 10 min, activity reached a plateau that was sustained for at least 30 min. In subsequent studies, blood was obtained 10 min after heparin administration.

Various amounts of heparin were administered to the same male subject to determine the optimal dose. These studies were performed at 2-day intervals. In four males a dose of 60 units/kg body wt was found to produce maximal glycerophospholipid hydrolysis activity (Fig. 2B). Heparin at a dose of 10 units/kg yielded only 45% of maximal activity for glycerophospholipid hydrolysis. The dose of 60 units/kg provided maximal activity for glycerophospholipid hydrolysis but activity decreased 20% or more at doses of 120 units/kg and above. Boberg (25), in studying triglyceride lipase activity in men, found a similar response to various levels of heparin.

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