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DRUG STANDARDS

Quantitative Fluorometric Determination of Pancreatic Lipase in Pharmaceuticals

S. S. WAGLE▲ and D. G. HOUSE

Abstract □ A rapid and reliable assay for pancreatic lipase is described. The method utilizes microquantities of enzyme and a fluorescent substrate. Among the many fluorescent substrates available, 4-methylumbelliferone laurate was chosen as the most suitable for this method. The rate of hydrolysis at different enzyme levels was determined at 25°. Interference due to nonspecific esterases and the effect of some pharmaceutical agents were evaluated. The fluorometric assay procedure was compared to the NF XIII potentiometric method. Pancreatic lipase in concentrations as low as 0.15 NF unit of activity could be detected. Various complex pharmaceutical preparations were compared using the fluorometric and the potentiometric methods. The fluorometric procedure was found to be reproducible and useful in determining content uniformity.

Keyphrases □ Pancreatic lipase—quantitative fluorometric determination in pharmaceuticals □ Fluorometry—quantitative determination of pancreatic lipase in pharmaceuticals □ Enzymes, pancreatic lipase—quantitative fluorometric determination in pharmaceuticals

For many years, pancreatic enzymes have been used as digestive aids. A number of pharmaceutical preparations contain pancreatic enzymes alone or in combination with other therapeutic agents. Many assay procedures have been devised to determine the potencies and activities of the lipolytic enzymes in these preparations. Aldridge (1) first described a manometric method utilizing carbon dioxide liberation from bicarbonate buffer to detect lipases in biological extracts. However, titrimetric measurement of acid liberation from natural fats was found to be more convenient (2, 3). Over the years, this procedure has been greatly modified and refined to improve its reliability and reproducibility (4). The potentiometric titration method, although reliable, is cumbersome and time consuming for routine analysis. This procedure also lacks the versatility needed for testing complex pharmaceutical preparations and ascertaining content uniformity.

Fluorometric procedures have a distinct advantage over other methods in that they are very sensitive. For a number of years, biochemists and clinical chemists detected minute amounts of esterase in microorganisms by utilizing fluorogenic substrates such as 1-naphthyl esters (5). Most fluorometric procedures for hydrolytic enzymes involve hydrolyzing a nonfluorescent substrate to a highly fluorescent product. 1-Naphthyl phosphate, a nonfluorescent substrate, is readily hydrolyzed by the enzymes, acid phosphatase and alkaline phosphatase, producing the free fluorescent product, 1-naphthol, which is then measured quantitatively by the fluorometric method. Similarly, umbelliferone and 4-methylumbelliferone are highly fluorescent compounds while their ester derivatives are nonfluorescent. These derivatives are ideal substrates for many enzymes (6). Recently, a number of investigators (6–8) reported new derivatives of fluorescein and 7-hydroxy-4-methylcoumarin to detect and locate minute quantities of lipase in plants

Table I—Relative Fluorescence of Fluorogenic Substrates and Their Hydrolysis Products

	Wavelength, nm.		Fluorescence Values: 1×10^{-5} *M* Solutions in pH 9.0, 0.05 *M* Phosphate Buffer		
	Excitation	Fluorescence	Substrate	Hydrolysis Product	Product/Substrate Ratio
Fluorescein dibutyrate	493	570	11	951	86.5
4-Methylumbelliferone laurate	380	450	29	397	13.7
1-Naphthyl acetate	335	450	11	26	2.36

Table II—Effects of Pharmaceutical Agents on Pancreatic Lipase-Catalyzed Hydrolysis of Fluorescein Dibutyrate and 4-Methylumbelliferone Laurate

	Relative Hydrolysis Rate, nmoles/min.				
	Control[a]	Sodium Taurocholate[b]	Dioctyl Sodium Sulfosuccinate[c]	1-Hyoscyamine Sulfate[d]	Trypsin[e]
Fluorescein dibutyrate	19.0	19.15	73.4	1.8	15.9
4-Methylumbelliferone laurate	42.83	43.0	44.1	43.6	13.5

[a] Control experiment contained 0.3 NF unit of lipase standard. [b] Sodium taurocholate, 5 mg./ml. [c] Dioctyl sodium sulfosuccinate, 25 mcg./ml. [d] Hyoscyamine sulfate, 50 ng./ml. [e] Trypsin, 1.2 NF units.

and biological fluids. The suitability of the fluorogenic substrates for quantitative analysis of pancreatic lipase in pharmaceutical preparations has not been investigated.

This paper reports a systematic study of some fluorogenic substrates and a simple fluorometric assay procedure for rapid and quantitative determination of lipase in pharmaceutical preparations.

EXPERIMENTAL

Apparatus—The rate of hydrolysis of the fluorogenic substrate is measured as the rate of increase in fluorescence and is simultaneously recorded by a fluorometer[1] with a recorder attachment. Clear fused Pyrex glass cells (10 × 10 × 65 mm.) were used as reaction vessels, and the instrument supplier's slit arrangement No. 1 was used throughout the work. The excitation and fluorescent wavelengths used for various substrates are listed in Table I.

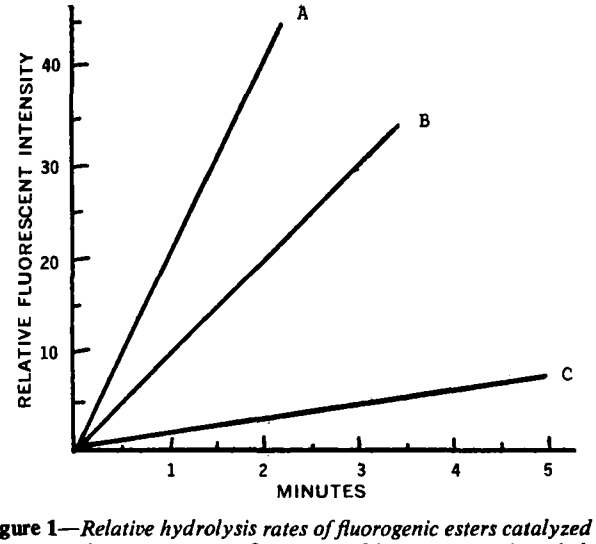

Figure 1—*Relative hydrolysis rates of fluorogenic esters catalyzed by pancreatic lipase. Key: A, fluorescein dibutyrate; B, 4-methylumbelliferone laurate; and C, 1-naphthyl acetate.*

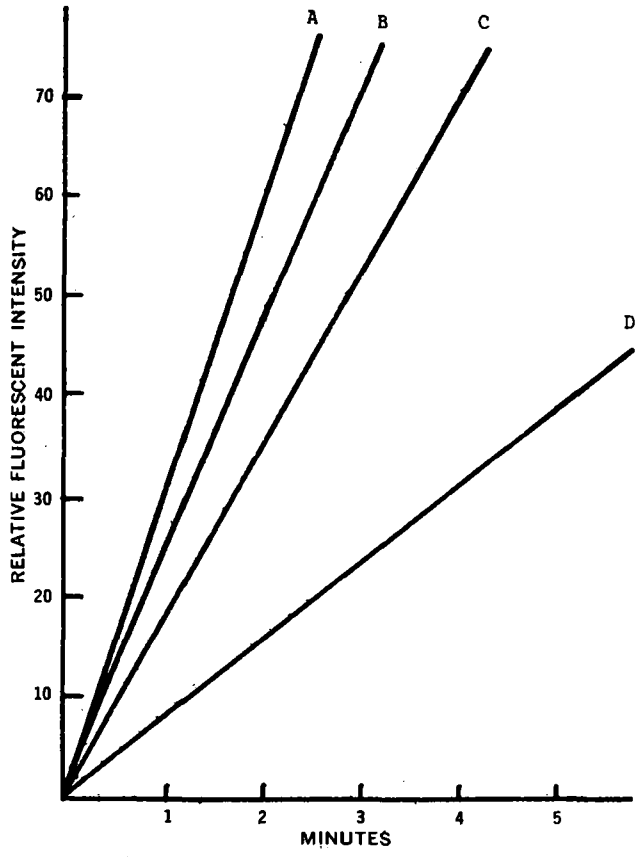

Figure 2—*Lipolysis of 4-methylumbelliferone laurate. Incubation mixture contained 5 × 10^{-5} mole of substrate and varying amounts of pancreatic lipase standard. Key: A, 0.6 NF unit; B, 0.45 NF unit; C, 0.30 NF unit; and D, 0.15 NF unit, in a total volume of 5 ml. of 0.05* M *phosphate buffer, pH 9.0, at 25°.*

Reagents and Chemicals—Stock solutions (1×10^{-2} *M*) of 1-naphthyl acetate[2], fluorescein dibutyrate[2], and 4-methylumbelliferone laurate[3] were prepared in 2-methoxyethanol[4]. These stock solutions are stable for at least 1 year when frozen. A fresh working solution (1×10^{-5} *M*) was made by diluting 1 ml. of the stock solution to 1 l. with 0.05 *M* disodium hydrogen phosphate buffer, pH 9.0.

Samples—At least 20 capsules (or tablets) were emptied into a beaker and mixed well, and a quantity equivalent to one capsule content was accurately weighed into a beaker and mixed thoroughly with 2–3 drops of glycerin. When the contents were thoroughly wet, 25 ml. of phosphate buffer (0.05 *M*) was added. The mixture was stirred for 5–10 min., quantitatively transferred into a centrifuge tube, and spun at 3000×*g* for 10 min. The supernatant liquid was transferred into a suitable flask and diluted with phosphate buffer to obtain a desired concentration of lipase activity.

Standard Solution—One hundred milligrams of the NF pancreatin reference standard (for protease and lipase activity) was accurately weighed and was treated in the same manner as the sample to obtain a desired concentration.

Other Pharmaceutical Agents—Dioctyl sodium sulfosuccinate USP, hyoscyamine sulfate NF, sodium taurocholate NF, and trypsin[5] were either dissolved or suspended in 0.05 *M* phosphate buffer, pH 9.0.

[1] Model 111, G. K. Turner.
[2] Eastman Kodak Co.
[3] Nutritional Biochemicals, Cleveland, Ohio.
[4] Aldrich Chemical Co., Milwaukee, Wis.

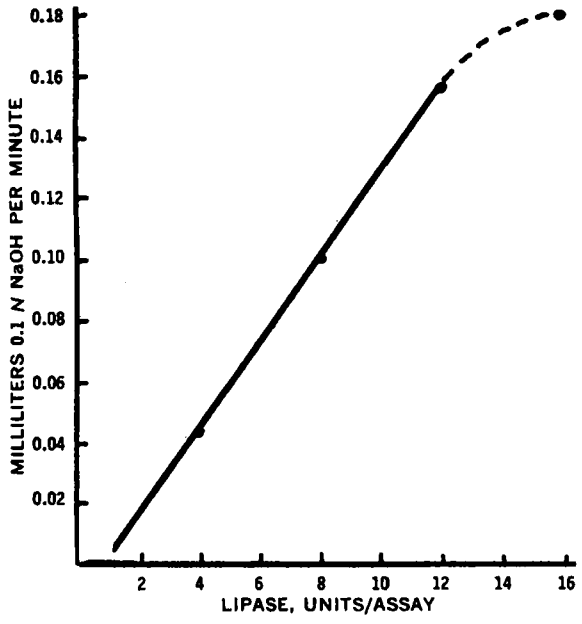

Figure 3—*Representative standard curve for pancreatic lipase by NF method.*

Procedure—Enzymatic reactions were conducted at 25° in the fluorometer cells. The reaction mixture contained 1 ml. of the substrate (1×10^{-5} *M*), with or without inhibiting or potentiating agents, and variable amounts of lipase (between 0.2 and 0.6 NF unit of activity per assay) in a total volume of 5 ml. The cell was placed immediately in a fluorometer, and the increase in fluorescence was recorded for the next 5–7 min. The rate of hydrolysis was calculated from the slope of the curve. For comparison, the potentiometric assay for lipase was performed as described in the second supplement of NF XIII (4).

RESULTS AND DISCUSSION

The effects of temperature and pH on lipolysis of fluorogenic substrates have been extensively studied by several investigators (6, 7, 11). In the present investigation, the hydrolytic reaction was conducted in phosphate buffer at pH 9.0 to reduce the possible deactivation of lipases by proteolytic enzymes which may be present in the formulations. No special precautions were necessary to control the cell temperature since the study indicated that the temperature of the reaction mixture varied less than a degree during the entire procedure.

Three fluorogenic esters were investigated as possible lipase substrates. Several important criteria, such as substrate sensitivity and specificity and enzyme inhibition and potentiation by pharmaceutical agents normally employed in the enzyme preparations, were tested and evaluated. To demonstrate hydrolysis at a very low enzyme concentration, the substrate should possess a relatively high fluorescence after hydrolysis. Table I shows the relative fluorescence of the three prospective fluorogenic substrates and their hydrolysis products. All fluorogenic substrates tested were hydrolyzed by lipase; however, fluorescein dibutyrate and 1-naphthyl acetate produced the highest and the lowest fluorescence, respectively, after hydrolysis and the latter exhibited the lowest hydrolytic rate (Fig. 1).

Table III—Comparative Analysis of Commercial Preparations by NF Method and Fluorometric Method[a]

Product	NF Method	Fluorometric Method[b]	Variation, %
A	16.49	16.8 ± 0.28	1.87
B	14.69	15.0 ± 0.40	2.11
C	20.80	22.2 ± 0.44	6.73

[a] Assay values presented in NF units. [b] Mean of at least six determinations.

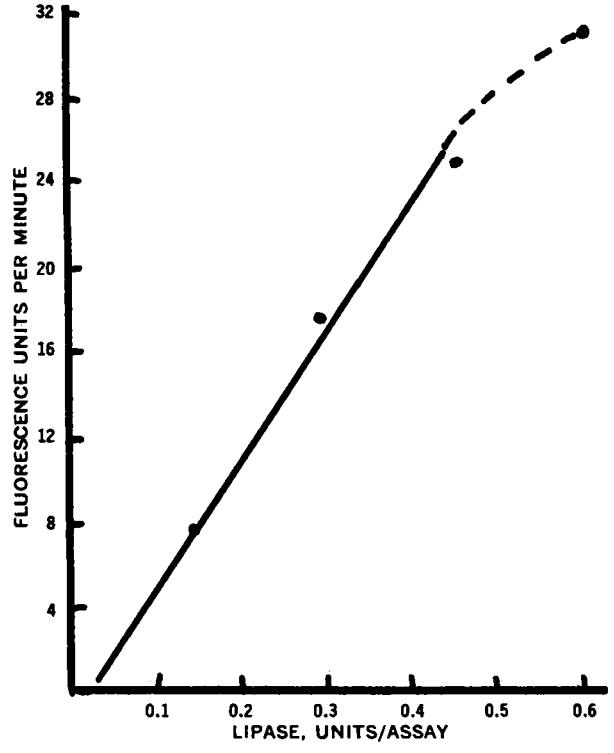

Figure 4—*Representative standard curve for pancreatic lipase by fluorometric method.*

It is imperative that minute quantities of pharmaceutical agents and excipients employed in the formulation have a minimal effect on the lipolysis of the substrate. The effects of some pharmaceutical agents and other interfering enzymes are summarized in Table II.

Trypsin is found in significant amounts in pancreatic extracts and possesses high esterase activity. Many substrates used in fluorometric assays are nonspecific and are hydrolyzed by both esterases and lipases. Therefore, a substrate that is specific for lipase will have a distinct advantage. Experiments revealed that fluorescein dibutyrate was readily hydrolyzed by both trypsin and lipase. 4-Methylumbelliferone laurate, on the other hand, was specifically hydrolyzed by lipase and was least affected by trypsin (Table II).

Some pharmaceutical and medicinal agents exhibit biological activity by virtue of their potentiating or inhibiting actions on enzyme catalysis (9). Bile salts and other surfactants are commonly used to enhance lipolysis of olive oil or other fatty esters (10). The data, however, indicate that sodium taurocholate had no potentiating effect on the rate of hydrolysis of fluorescein dibutyrate or 4-methylumbelliferone laurate. Another potent surfactant, dioctyl sodium sulfosuccinate, at 25-mcg./ml. concentration, enhanced the lipolysis of fluorescein dibutyrate 400%, but no such effect could be demonstrated on the hydrolysis of 4-methylumbelliferone laurate. Belladonna alkaloids, particularly hyoscyamine sulfate, are frequently formulated in combination with digestive enzymes. Hyoscyamine sulfate inhibited the enzymatic hydrolysis of fluorescein dibutyrate but had no apparent effect on the hydrolysis of 4-methylumbelliferone laurate. The data summarized in Table II clearly suggest that 4-methylumbelliferone laurate is a preferable substrate for fluorometric determination of lipase.

Figure 2 shows a typical continuous recorder tracing of hydrolysis of 4-methylumbelliferone laurate at different concentrations of NF lipase standard. The slope of the curve thus represented the rate of hydrolysis. This, when plotted against the concentration of enzyme, provided a standard curve for quantitative estimation of lipase. Using the NF lipase standard, comparison was made of the rates of hydrolysis obtained by the NF method and the fluorometric method. The data show that essentially the same linear rate was obtained using either method (Figs. 3 and 4). The fluorometric method, however, was 10–20 times more sensitive than the potentiometric procedure.

Comparative analyses were conducted on commercial preparations using potentiometric and fluorometric methods. All products

tested contained, in addition to lipases, other hydrolytic enzymes of pancreatic, plant, or microbial origin mixed with lactose and smaller quantities of lubricants. The data were analyzed statistically, and excellent agreement between the two methods was observed (Table III). The reproducibility of the method was evaluated by performing repeated analysis of the same enzyme solution. The precision of the method was determined from the variability obtained upon repeated analysis of lipase standard or samples. A standard deviation of 0.76, with a coefficient of variation of 3.05%, was obtained.

CONCLUSION

The advantages of a fluorometric procedure are many. In contrast to earlier methods, the fluorometric assay is more sensitive and requires smaller amounts of substrate and enzyme. The lipolytic measurements can be made at such a great dilution that interfering enzymes and other excipients are frequently diluted out. 4-Methylumbelliferone laurate was found to be a most suitable substrate for the fluorometric assay, because it was unaffected by a number of pharmaceutical agents and enzymes tested.

The method shows a high degree of precision and accuracy. The procedure is simple and requires little preparation. The substrate is stable under the experimental conditions and can be stored frozen for long periods. The method may be valuable in determining content uniformity of pharmaceutical preparations containing lipase.

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Sensitive Colorimetric Determination of Isoniazid

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Abstract □ A sensitive colorimetric method was developed for the assay of isoniazid. The method is based on measuring the absorbance at 467 nm. of the orange-colored product produced by the interaction of isoniazid with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole in the presence of sodium tetraborate in absolute methanol. The Beer–Lambert law was obeyed over the concentration range of 0.3–5 mcg./ml, and the color was stable for at least 90 min. The coefficient of variation as determined on nine replicate samples containing 2 mcg. isoniazid/ml. was 0.87%. Applied to the assay of isoniazid tablets and syrups, the method gave results comparable to those obtained by the USP XVIII method. It also afforded improvements in sensitivity, ease, and speed over the official method. It was established that the major metabolites of isoniazid (acetylisoniazid and isonicotinic acid) produced no color with the reagent under the proposed experimental conditions.

Keyphrases □ Isoniazid—colorimetric analysis, compared to compendial method □ Colorimetry—analysis, isoniazid, compared to compendial method

Isoniazid (isonicotinyl hydrazine, I) is a remarkably effective drug which is now considered a primary drug for the chemotherapy of tuberculosis. It is not surprising that the determination of this drug has been, and is still, the subject of much investigation (1, 2). The need for further investigation is evidenced by the fact that many of the assay procedures reported appear not to be very satisfactory. For example, the current official assay method for isoniazid employs an iodometric titration in the presence of sodium bicarbonate (3). This method, originally developed by Canbäck (4), involves a slow reaction; 90 min. is needed for the completion of the reaction before a back-titration of the excess iodine can be performed. In addition, at several stages during the assay period, loss of iodine by volatilization can occur. The USP XVIII method for the assay of isoniazid tablets is time consuming and laborious; *at least* 2.5 hr. is required for the extraction of the drug from the tablet mass, evaporation of the extract, and completion of the reaction (5).

Several methods for the analysis of isoniazid in the microgram range have been developed using 1-chloro- or 1-fluoro-2,4-dinitrobenzene as the reagent (6, 7). Reisch *et al.* (8) recently reported the use of a similar reagent for visualization of isoniazid spots on thin-layer chromatoplates. This reagent, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (II)[1], gave a colored product

[1] NBD Chloride, Aldrich Chemical Co., Milwaukee, Wis.