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A RAPID MODIFIED GAS CHROMATOGRAPHIC ASSAY FOR ESTERASE ACTIVITY

SHUNSUKE KAGEHIRA, SATOSHI KAWAI and TAKEO OHNO
Gifu College of Pharmacy, Mitahora, Gifu (Japan)
and
HIDEKO ISHIHARA and HIROH IKEZAWA
Faculty of Pharmaceutical Science, Nagoya City University, Nagoya (Japan)
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SUMMARY

A rapid modified procedure for the gas chromatographic determination of esterase activity was studied. Aliphatic esters such as ethyl *n*-butyrate, *n*-propyl *n*-butyrate, *n*-butyl *n*-butyrate and *n*-amyl *n*-butyrate were used as substrates and acetone was chosen as the most suitable solvent for dissolving the substrates in order to avoid alcoholysis. The enzyme reaction was started in a mixture of 0.03 *M* phosphate buffer, pH 7.90, containing an adequate amount of an internal standard and the substrate solution in acetone. At definite intervals, an aliquot of the reaction solution was injected directly on to a gas chromatograph and the alcohols produced were separated.

INTRODUCTION

Reports dealing with gas chromatographic (GC) methods for measuring enzymatic activity are not numerous, but a GC method generally has the following merits compared with fluorimetry or colorimetry: (1) the products or the remaining substrates in the reaction mixture are specifically measured, so the values obtained are more reliable because there is little interference by various co-existing substances; (2) natural substrates can be used under conditions similar to those of enzyme reactions in a living organism; (3) this method makes it possible to study enzyme reactions with mixed substrates, offering an advantageous technique for the simultaneous determination of some co-existing enzymes, and the study on substrate specificity and kinetics of the enzyme or the differentiation of iso-enzymes; and (4) comparison of the enzyme reaction patterns obtained with mixed substrates may be applicable to clinical diagnosis, without an absolute determination of individual products.

From these standpoints, Ikezawa and co-workers[1,2] reported a GC assay of esterase activity, where the properties of esterases in the presence of high concentrations of alcohols were studied. In this paper, a more rapid and simple method for the GC assay of esterase activity is described.

EXPERIMENTAL

Materials

Substrate solutions. Ethyl *n*-butyrate (1000 μmoles/ml), *n*-propyl *n*-butyrate (400 μmoles/ml), *n*-butyl *n*-butyrate (500 μmoles/ml) and *n*-amyl *n*-butyrate (125 μmoles/ml) solutions were prepared in acetone.

Buffer solution. A 0.03 *M* sodium phosphate buffer of pH 7.90 was used.

Internal standard (IS) solution. Concentrations of 2% dimethylformamide (DMF), 2% dioxane and 2% methyl Cellosolve (MC) were used in the buffer solution.

Enzymes. Pig liver esterase (carboxyl ester hydrolase, E.C. 3.1.1.1) was a commercial product (specific activity for *n*-propyl *n*-butyrate, 100 U/mg) purchased from Boehringer, Mannheim, G.F.R. This preparation contained 224 units/ml of enzyme activity, and was diluted 100-fold with 0.03 *M* phosphate buffer (pH 7.90) for use. Rat liver acetone powder was prepared by washing homogenized rat liver twice with 20 volumes of acetone and the residue was kept in a desiccator. Before use, 1 g of the residue was extracted with 3 ml of the buffer solution, the mixture was centrifuged and the supernatant was used.

Apparatus and conditions

A Shimadzu GC-4APF gas chromatograph equipped with a flame ionization detector was used. A 3-m stainless-steel column packed with 20% polyethylene glycol (PEG) 20 M on 80–100 mesh Celite 545 (AW DMCS) was used. The carrier gas (nitrogen) flow-rate was 40 ml/min, the hydrogen flow-rate 44 ml/min, the detector temperature 250°, the injection temperature 200° and the chart speed 0.5 cm/min.

Standard procedure

Table I shows the standard procedure for the assay of esterase activity. System A is appropriate for determinations on sample solutions with activity higher than 0.5 U/ml, such as pig liver esterase or rat liver acetone powder, while system B is suitable for sample solutions with lower activities such as serum or urine. The mixed solution containing buffer, IS and substrate as described in Table I was pre-incubated at 30° for 5 min and the enzyme reaction was initiated by addition of enzyme. At the start and at definite intervals, 1–2-μl aliquots of reaction solution were subjected to gas chromatography. The ratio of the peak height of the alcohol produced to that of IS was plotted against the reaction time, then the initial rates of hydrolysis of substrate

TABLE I

STANDARD PROCEDURE

Solution added	*System A**	*System B***	*Conditions*
Buffer solution (ml)	0.750	0.225	30°, 5 min, pre-incubation
IS solution (ml)	0.100	0.050	
Substrate solution (ml)	0.050	0.025	
Sample solution (ml)	0.100	0.200	30°, incubation

* System A: for pig liver esterase or rat liver acetone powder.
** System B: for serum and urine.

(rate of production of alcohol) was calculated from a calibration graph and converted into units of esterase activity. One unit of esterase is expressed as the amount that will transform 1 μmole of *n*-propyl *n*-butyrate per minute.

Calibration graph

The following solutions of alcohols were prepared in acetone: ethanol, 100 μmoles (4.6 mg)/ml; *n*-propanol, 200 μmoles (12.0 mg)/ml; *n*-butanol, 150 μmoles (11.1 mg)/ml; and *n*-amyl alcohol, 150 μmoles (13.2 mg)/ml.

Five alcohol solutions were prepared by diluting each of the above solutions with acetone to 1:5, 2:5, 3:5, 4:5 and 5:5 concentrations. To each of five test-tubes, 0.85 ml of 0.03 *M* phosphate buffer (pH 7.90), 0.05 ml of the diluted alcohol solution and 0.1 ml of IS solution were added. An aliquot of the mixture was injected into the gas chromatograph and the ratio of alcohol to IS was plotted aginst the amount of alcohol in the solution.

Calibration graphs for alcohols are linear up to the amount mentioned above.

RESULTS AND DISCUSSION

PEG was chosen as a suitable column packing for the GC separation of substrate and the corresponding alcohol, because it gave sharp symmetrical peaks and good separations. Fig. 1 shows a gas chromatogram of some esters and alcohols. Under these conditions, free fatty acids liberated from the substrate do not give any peaks.

From preliminary experiments, some suitable combinations of substrate, IS and GC conditions were chosen, as shown in Table II.

Solvent and buffer

An organic solvent was added to the reaction medium in order to aid the dis-

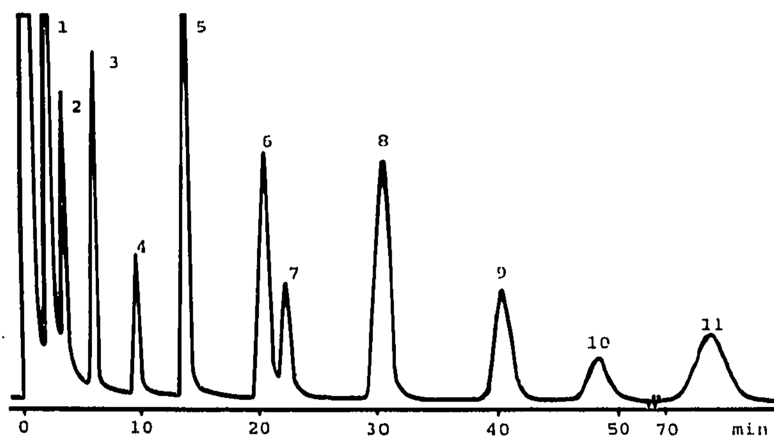

Fig. 1. GC separation of some esters and alcohols using a 3-m column packed with Celite 545 coated with PEG (20%, w/w) at 68°. 1 = Methanol; 2 = ethanol; 3 = *n*-propyl acetate; 4 = *n*-propanol; 5 = *n*-butyl acetate; 6 = *n*-propyl *n*-butyrate; 7 = *n*-butanol; 8 = cumen (added as an IS); 9 = *n*-butyl *n*-butyrate; 10 = *n*-amyl alcohol; 11 = *n*-amyl *n*-butyrate.

TABLE II

GC CONDITIONS FOR ASSAY OF ENZYMATIC REACTION

Substrate n-butyrate	*Column temp. (°C)*	*Retention time (min)*		*IS*	*IS retention time (min)*
		Alcohol	*Ester*		
Ethyl	75	1.50	4.30	Dioxane	5.60
n-Propyl	88	2.87	4.20	Dioxane	5.92
n-Propyl	110	1.25	2.73	Methyl Cellosolve	4.00
n-Butyl	140	1.70	3.10	DMF	6.20
n-Amyl	125	4.80	7.55	Dioxane	2.15

solution of the substrate. Alcohols resulted in alcoholysis of the substrate, as described in the previous paper[2]. Of the solvents tested, acetone was chosen as a suitable solvent instead of alcohol, because it can mix with water and does not disturb the separation on the chromatograms.

Fig. 2 illustrates the effect of acetone concentration on the hydrolytic activity of pig liver esterase. A marked decrease in hydrolytic activity of the enzyme was observed with increasing acetone concentration in the reaction medium, so the acetone concentration was fixed at 5%. The optimum pH of pig liver esterase was 7.90 and the esterase activity was little affected by the concentration of buffer solution at that pH. In the previous paper, Ishihara *et al.*[2] used alcohols as solvents for dissolving the substrate, and deoxycholate was added as a substrate emulsifying agent. In this work, it was found that by using acetone as the organic solvent, no addition of deoxycholate was necessary.

Substrate concentration

Using pig liver esterase, the correlation between the amount of substrate and esterase activity was tested. The following substrate concentrations gave the maximum

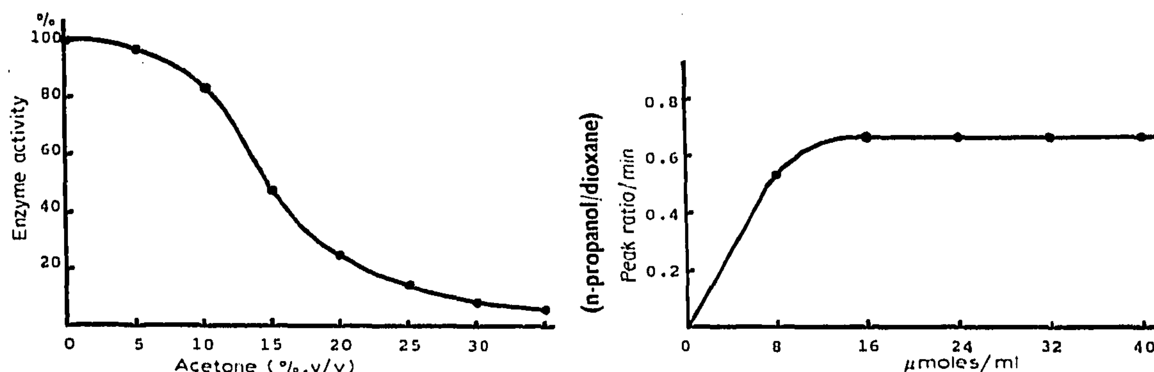

Fig. 2. Effect of acetone content on the enzyme activity.

Fig. 3. Effect of *n*-propyl *n*-butyrate concentration on pig liver esterase activity.

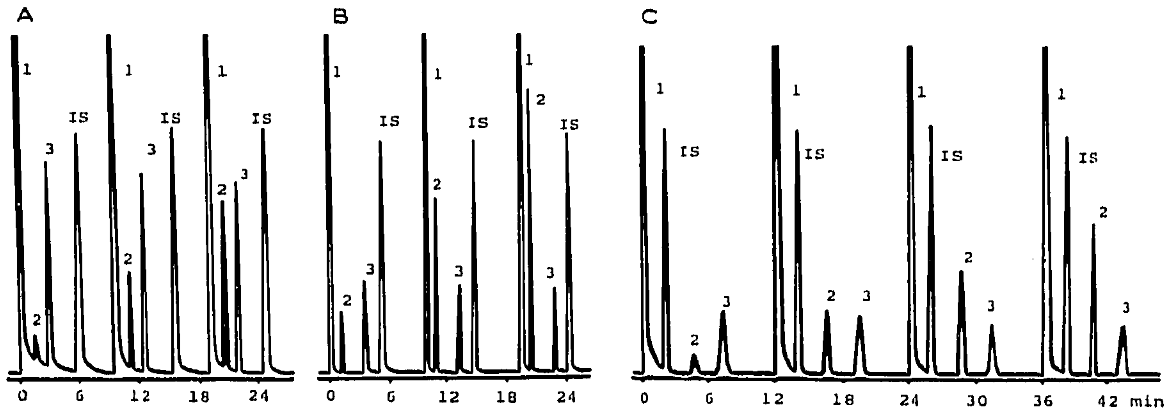

Fig. 4. Some examples of GC separations where pig liver esterase activity was assayed using *n*-butyl *n*-butyrate (A), *n*-propyl *n*-butyrate (B) and *n*-amyl *n*-butyrate (C) as substrate. A: 1 = acetone; 2 = *n*-butanol; 3 = *n*-butyl *n*-butyrate; IS = DMF; column temperature, 140°. B: 1 = acetone; 2 = *n*-propanol; 3 = *n*-propyl *n*-butyrate; IS = dioxane; column temperature, 88°. C: 1 = acetone; 2 = *n*-amyl alcohol; 3 = *n*-amyl *n*-butyrate; IS = dioxane; column temperature, 125°.

activity for 0.1 ml of 100-fold diluted pig liver esterase: 16.00 μmoles/ml of *n*-propyl *n*-butyrate (Fig. 3), 25.00 μmoles/ml of *n*-butyl *n*-butyrate and 3.10 μmoles/ml of *n*-amyl *n*-butyrate. Therefore, substrate concentrations greater than those described above are adequate for the measurement of esterase activity.

Fig. 4 illustrates some examples of GC separations where the activity of pig liver esterase was assayed using *n*-butyl *n*-butyrate, *n*-propyl *n*-butyrate or *n*-amyl *n*-butyrate as a substrate. Spontaneous hydrolysis of substrates was observed in the absence of enzyme. The time course of this non-enzymatic hydrolysis in the control was linear, as shown by a dotted line in Fig. 5. Thus, the rate of hydrolysis by the enzyme is calculated by subtracting that of non-enzymatic hydrolysis from the total rate (full line).

The relationship between the amount of alcohol produced and pig liver esterase concentration was found to be linear up to 20 μl of the diluted enzyme solution (1:100).

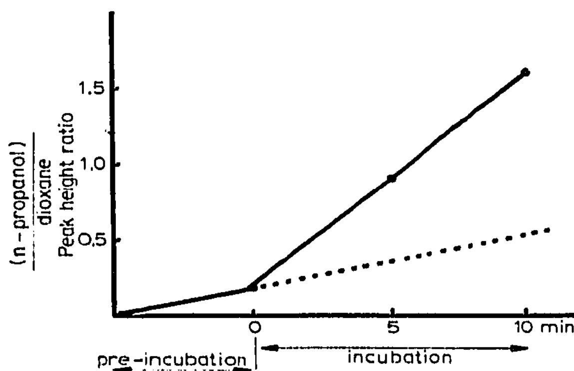

Fig. 5. An example of enzymatic hydrolysis using *n*-propyl *n*-butyrate as a substrate. Broken line, non-enzymatic hydrolysis; full line, total hydrolysis.

TABLE III

ENZYMATIC HYDROLYSIS PATTERN

Values are expressed as activity relative to that of pig liver esterase with *n*-propyl *n*-butyrate (taken as 100). Values in parentheses are activities Units per millilitre.

Enzyme	*Ethyl n-butyrate*	*n-Propyl n-butyrate*	*n-Butyl n-butyrate*	*n-Amyl n-butyrate*
Pig liver esterase	86	100 (2.24)	67	50
Rat liver acetone powder	7	33 (0.75)	22	11
Urine*	0.5	0.75 (0.017)	0	0

* Urine esterase activity was assayed by System B.

The hydrolytic activities of some samples were measured with four substrates and the results are shown as relative activity or units per millilitre in Tables III and IV. Table III shows the results obtained with pig liver esterase, rat liver acetone powder extract and urine. Table IV shows the results with some specimens of human serum. The esterase activity in urine was extremely low, so it was very difficult to measure it. As shown in Table III, the fact that the relative activity of pig liver esterase with *n*-propyl *n*-butyrate > ethyl > *n*-butyl > *n*-amyl *n*-butyrates was in good agreement with that in 3.5 *M* methanol in the previous paper[2]. Of these esters, *n*-propyl *n*-butyrate was the best substrate for the measurement of esterase activity.

Mixed substrates

When two kinds of esters were used as mixed substrates, the resulting products could be measured simultaneously by gas chromatography. Fig. 6 illustrates a typical chromatogram with mixed substrates containing *n*-propyl and *n*-amyl *n*-butyrates. This method with mixed substrates is expected to offer an interesting means for the simultaneous determination of some co-existing enzymes and for studies on the kinetics and substrate specificity of the enzyme or the differentiation of iso-enzymes.

The method described here has the following merits. (1) Acetone was used as a solvent to dissolve the substrates instead of an alcohol, such as methanol or ethanol, thereby simplifying the enzyme reaction by avoiding alcoholysis. (2) Prior isolation of the products was omitted and an aliquot of reaction mixture was injected directly

TABLE IV

ACTIVITY OF SERUM ESTERASE

Values are expressed as relative activities obtained by System B. Values in parentheses are activities in Units per millilitre.

Serum	*Sex*	*Age*	*n-Amyl n-butyrate*	*n-Propyl n-butyrate*
1	Male	23	14.1	100 (0.105)
2	Male	24	13.3	100 (0.133)
3	Female	22	9.9	100 (0.145)
4	Male	24	13.3	100 (0.093)

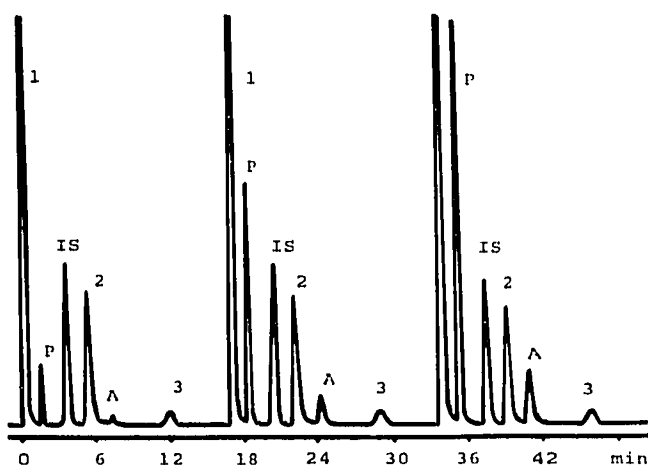

Fig. 6. Enzymatic reaction with mixed substrates using pig liver esterase. 1 = Acetone; P = *n*-propanol; **IS** = dioxane; 2 = *n*-propyl *n*-butyrate; A = *n*-amyl alcohol; 3 = *n*-amyl *n*-butyrate. Column temperature, 110°.

on to the column, and consequently the procedure is simplified and the precision of the assay improved. In addition, this method makes it possible to trace directly the time course of the enzyme reaction using the same reaction mixture, without stopping the enzyme reaction by extracting with *n*-hexane. (3) A sufficient amount of substrates can be added to make the enzyme reaction constant, because the method is based on the measurement not of the remaining substrates, but of the corresponding products of the enzyme reaction, which is also expected to improve the precision of the assay.

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