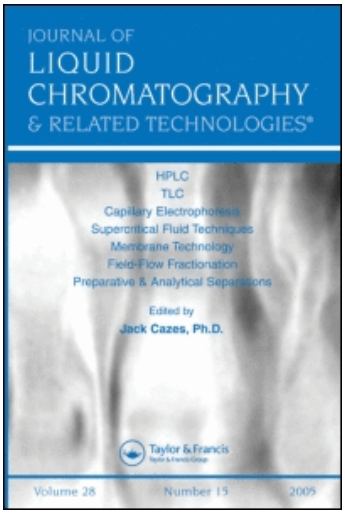

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A SEMICONTINUOUS ASSAY OF INHIBITION OF CYCLIC-AMP PHOSPHODIESTERASE BY BENZO[c]PHENANTHRIDINE ALKALOIDS

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ABSTRACT

An HPLC apparatus to trace the reaction semicontinuously was devised and applied to the kinetic study of an enzyme reaction. From the thermostated solution in which the reaction proceeds continuously, an aliquot of the solution was removed and submitted to HPLC automatically and repeatedly. Thus, the change of concentrations of reactant and/or product was determined at a definite short interval. The inhibitory action of cyclic-AMP phosphodiesterase (PDE) by several benzo[c]phenenthridine alkaloids was examined. From 1/S - 1/V (Lineweaver-Burk plot) sanguinarine and chelerythrine, which were both potent bacteriocidal alkaloids, were found to strongly inhibit PDE competitively. Kinetic parameters of the enzyme reaction such as Michaelis constant and inhibition constants were obtained.

INTRODUCTION

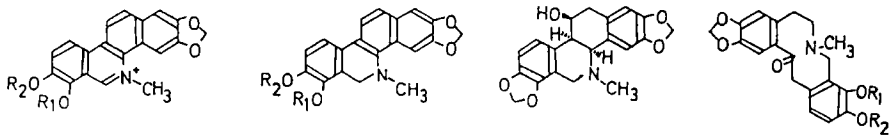
The HPLC studies of enzyme reactions have extensively been carried out (1). In most of these studies the enzyme reaction was

initiated under proper conditions, and after the lapse of some time the enzyme was deactivated and the reaction was halted by some way such as boiling or addition of acids or bases. The concentrations of product and/or substrate were then analyzed by HPLC. In such an approach (end point method), in order to elucidate the mode of inhibition and to obtain kinetic parameters such as inhibition constant, it is necessary to prepare a lot of samples which contain different concentrations of substrate and sometimes enzyme. Such measurements are not only time-consuming but necessitate rather large amount of materials, which is not suitable when samples tested are natural origin and consequently available only a very minute amount. If the progress of the enzyme reaction can be monitored continuously, kinetic parameters will be obtained easily by the use of a very small amount of sample within short time. In the present study an apparatus suitable for such measurements was constructed, and applied to the inhibiting action of 3':5'-cyclic nucleotide 5'-nucleotidohydrolase (phosphodiesterase EC 3. 1.4. 17, hereafter abbreviated as PDE) by several pharmacologically active benzo[c]phenanthridine alkaloids.

It is well known that many of the pharmacologically active substances inhibit PDE activity, and vice versa. Thus, the inhibition of PDE has widely been used one of the first screening methods for substances of both natural and synthetic origin which may be available as medicine (2). The most versatile assay method is to utilize radioisotope kit commercially available, and an HPLC method is also applied (3,4). These methods all measure only one value of the concentration of substrate (or product) after halting the reaction. Therefore, we tried in this report to develop the method of semicontinuous measurements.

MATERIALS AND METHODS

Reagents. Adenosine cyclic 3',5'-(hydrogen phosphate) (CAMP) and adenosine 5'-phosphate (AMP) were purchased from



sanguinarine 1 $R_1 + R_2 = CH_2$
chelerythrine 2 $R_1 = R_2 = CH_3$

dihydrosanguinarine 6 $R_1 + R_2 = CH_2$
dihydrochelerythrine 7 $R_1 = R_2 = CH_3$

(+)-chelidonine 5

protopine 3 $R_1 + R_2 = CH_2$
allocryptopine 4 $R_1 = R_2 = CH_3$

Fig. 1 Alkaloids tested

Yamasa Shoyu Co., Ltd., and both were guaranteed to be > 98 % pure. Beef heart PDE was supplied by Boehringer Mennheim Yamanouchi Co., Ltd., which contained about 2.5 U of enzyme per 1 cm^3. Alkaloids of benzo[c]phenanthridine and protopine type used were all plant origin: Sanguinarine 1, chelerythrine 2, protopine 3, and allocryptopine 4 were isolated from Macleaya cordata. Chelidonin 5 is a major alkaloid in Chelidonium majus. They are all obtained from plant materials. Reduction products of 1 and 2 (6 and 7, respectively) were prepared by the reaction of corresponding alkaloids with $NaBH_4$.

Apparatus. Figure 2 shows a block diagram of the apparatus constructed. The solution in a test tube themostated was sucked into a sample loop of an autoinjector (Model CRA-16, Chemco. Co., Ltd.) by a peristaltic pump at the rate about 0.2 cm^3 per 0.1 min. The flow path was then changed automatically and an aliquot of the solution was injected. After analyzing for some time, the flow path was changed again. Separation of CAMP, AMP and alkaloid to be tested was achieved on Nucleosil 10-DMA column (150 x 4.6 mm i.d.) by the eluent of acetonitrile-0.05 M KH_2PO_4 (1:1) at the flow rate of 3.0 cm^3/min. Under these conditions CAMP and AMP were separated and analyzed within 2 min. Alkaloids tested as well as various other substances were eluted

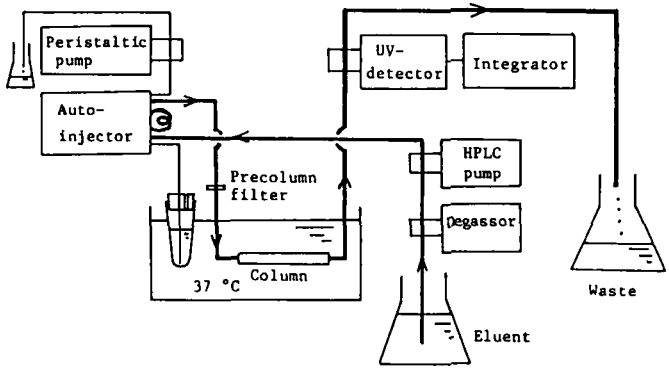


Fig. 2 Block diagram of the apparatus

without retaining on the column, and did not ovelap on CAMP and AMP. Consequently, the progress of the reaction could be monitored at an interval of 2 min. In the present study, the temperature was set either at 37 °C or 25 °C. The column was plunged into the bath themostated to keep the column at a regulated temperature. Other HPLC apparatus was the combination of the following units: Model 510 pump (Waters Associates); Model 319-A UV detector (Soma Kogaku Co., Ltd.); Chromatopak CR3A data processor (Shimadzu Co., Ltd.); precolumn module (Waters Associates) equipped with a precolumn filter or Guard-Pak precolumn cartridge (Resolve CN). The use of precolumn filter, or more desirable precolumn cartridge, effectively prevented the degradation of the column which may be caused by the repeated injection of macromolecular enzymes.

Procedures. To a test tube thermostated at 37 °C (or 25 °C), 0.2 cm^3 of 5 mM CAMP, 4.7 cm^3 of 3 mM $MgSO_4$ in 50 mM tris buffer (pH = 7.3) and 0.05 cm^3 of diluted dimethylsulfoxide solution of alkaloid to be tested were placed and incubated at 37 °C (or 25 °C) for more than 15 min. During that interval standard CAMP solution, which contained 0.2 mM of CAMP in tris buffer, was

analyzed for a few times and thus the peak height (or area) corresponding to initial concentration of CAMP was measured. The optimum pH of PDE is rather high (near pH = 8), but when pH of the reaction solution exceeded 7.5, magnesium hydroxide tended to precipitate, resulting trouble in HPLC analysis. Thus the pH of the solution was adjusted at 7.3. An aliquot (0.05 cm^3) of beef heart PDE, which was diluted to 1/3 by tris buffer, was then added by micro syringe, the mixture was shaken vigorously, and HPLC measurements were started. The volume of sample loop was chosen as 0.02 cm^3 and thus the initial amount of CAMP was about 1.3 μg. The volume of the sample loop could be increased to about 0.05 cm^3 without broadening of each peak. Determination of about 200 ng of CAMP and AMP was possible without the decrease of precision of the analysis. Threfore, it is possible to decrease the initial concentration of CAMP up to about 0.01 mM or lower, if necessary. Such measurements reduce the amount of inhibitor to be tested. In the present study, however, the concentration and smaple volume were set at 0.2 mM and 0.02 cm^3, respectively.

Calculation of reaction velocity. In the present measurements the decrease of the concentrations of substrate was determined at a definite interval. As shown in Fig. 3, the reaction velocity V_i at P_i should be the gradient of the reaction curve at P_i, which will be approximated by the following equation, provided that the interval of the measurements Δt was chosen to be small. The gradient at P_i can be approximated as the tangent of the triangle $P_{i-1}P_{i+1}Q_i$.

$$V_i = (-dS/dt)_i = -(S_{i+1} - S_{i-1})/(t_{i+1} - t_{i-1})$$
$$= -(S_{i+1} - S_{i-1})/2\Delta t$$

Thus, several set of values, S_i and t_i, were obtained by a single measurement. Consequently, from $1/S - 1/V$ (Lineweaver-Burk) plot, kinetic parameters will be obtained.

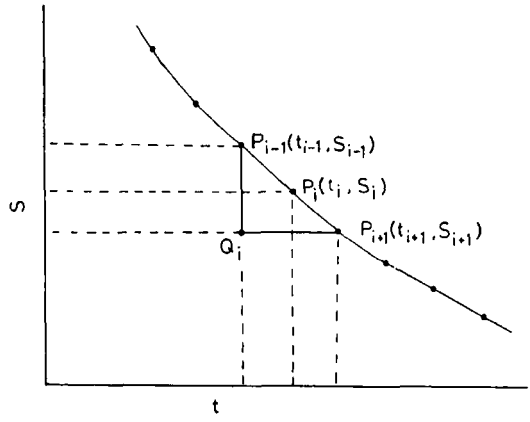


Fig. 3 Method for the determination of reaction velocity

RESULTS AND DISCUSSION

HPLC chromatograms and 1/S - 1/V plot. Figures 4(a) and (b) show example of chromatograms in the absence and presence of the inhibitor, respectively. With the lapse of time the peak of CAMP with the shorter retention time decreased while that of AMP with the longer retention time grew gradually. In Fig. 4(a), the inhibitor was not added and consequently the reaction was rather fast. The injection was repeated at 2 min interval. When 0.02 mM of 1, which amounted to 1/10 concentration of substrate in molar ratio, was added, the reaction rate was reduced about 30 % as depicted in Fig. 4(b). The peak areas and heights of CAMP were found to give linear calibration graphs within wide range. Consequently concentration of residual CAMP was easily calculated. In the present study, measurements were made 16 to 40 min in accordance with the reaction rate, during which 7 to 19 sets of values of S_i and V_i were obtained. Thus, Lineweaver-Burk plots were obtained.

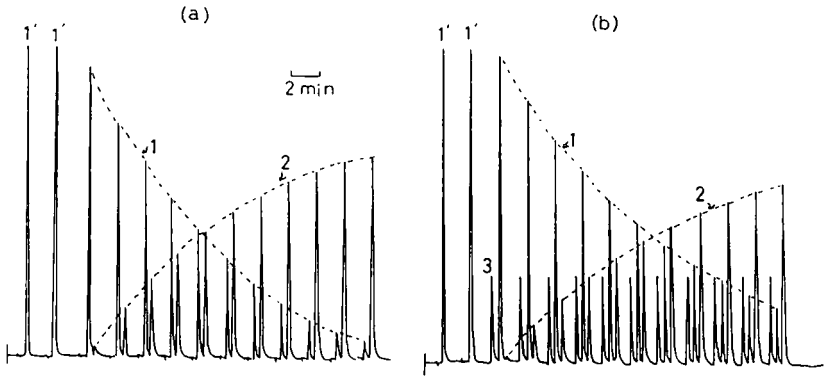


Fig. 4 Example of semicontinuous chromatograms in the absence (a) and presence (b) of the inhibitor
1 CAMP, 2 AMP, 3 0.02mM sanguinarine, 1' 0.2 mM CAMP (initial concentration)

Effect of the Amount of Enzyme. The rate of the enzyme reaction in the absence of inhibitor was observed by adding different amount of the enzyme at 37 and 25 °C. The reaction rate, of course, was faster at 37 °C than at 25 °C. By the method mentioned above, 1/S - 1/V plots were drawn at both temperatures. Figures 5(a) and 5(b) designate the 1/S - 1/V plot at 37 and 25 °C, respectively. In both cases, lienar 1/S - 1/V plots were obtained, and these graphs crossed at a point on horizontal axis, the reciprocal value of which should be Michaelis constant K_m. The maximum velocity of the reaction V_{max} was the reciprocal value of the intercept on vertical axis, which was also calculated. From V_{max} values, the micro molar amount of CAMP convertable to AMP at V_{max} per minute was calculated both at 25 °C (A) and 37 °C (B), which are shown in Table 1. In this table, enzyme unit of PDE added was calculated and was also shown(A'). Since an enzyme unit of this enzyme is defined as the amount of PDE that will catalyze the transformation of a micro molar amount of CAMP to AMP under optimum conditions, this value

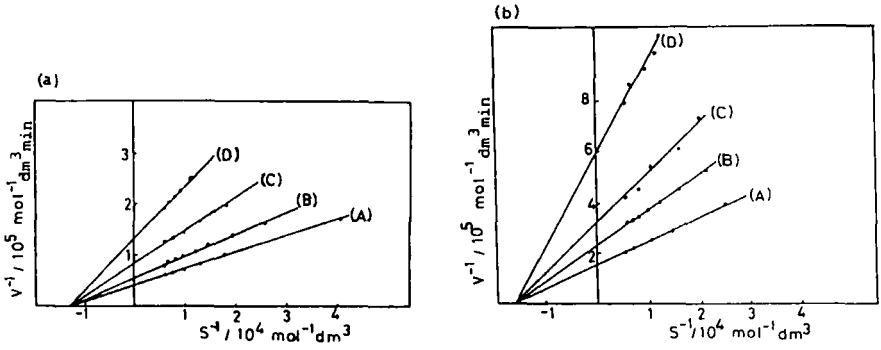


Fig. 5 Effect of enzyme amount on the reaction rate
(a) 37 °C, (b) 25 °C
Enzyme content: (A) 0.042 U, (B) 0.029 U,
(C) 0.021 U, (D) 0.013 U

Table 1 Dependence of the reaction rate on the PDE amount

PDE added / 10^{-2} U (A')	Amount of CAMP convertable to AMP at V_{max} / 10^{-8} mol min^{-1} 25 °C (A)	37 °C (B)
4.2 (100)	3.1 (100)	11.6 (100)
2.9 (70)	2.1 (67)	8.9 (70)
2.1 (50)	1.5 (48)	5.9 (51)
1.3 (35)	0.9 (30)	3.8 (33)

(A') should be close to (A) as long as guaranteed value of the enzyme purchased is correct. Both values agree rather well as shown in the table. Furthermore, the reaction rates approximately increased about 4 times with 12 °C rise in temperature, which seems reasonable in kinetics. The Michaelis

constant K_m was determined to be 60 μM and 70 μM at 25 and 37 °C, respectively, which is in good agreement with the data reported previously (5,6) because K_m of beef heart PDE reported previously was about 70 μM. These results suggest that the present method gives reliable kinetic data for the enzyme reaction. Hereafter, the experiments were carried out at 37 °C becuase most of the previous studies on inhibitory action of PDE had been carried out at 37 °C.

Inhibition by papaverine. Before applying the present method to alkaloids, we examined the inhibition by papaverine 8. Several substances have been used as a reference of PDE inhibition, and 8 belongs to one of these. In the present study the use of 8 as a reference seems to be best by the reason not only 8 is most frequently used but also 8 structurally resembles alkaloids to be tested. Alkaloids tested as well as 8 belong to benzylisoquinoline alkaloids and therefore rather structurally resemble.

It was established that 8 competitively inhibit PDE activity strongly (7). Figure 6 designates the 1/S - 1/V plot of 8 at 37 °C. The present data show that 8 inhibits PDE competitively as anticipated. The inhibition constant of 8 was determined to be 2×10^{-5} M, which is rather in good agreememt with the data previously reported (7).

Inhibition by alkaloids. The present method is then applied to the inhibition of PDE by a few benzo[c]phenanthridine alkaloids and related compounds. Sanguinarine 1 and chelerythrine 2 are potent bactericidal alkaloids (8) in Macleaya cordata, the activity of 1 being more potent than 2. Due to strong bactericidal activity of 1, the plant extract of Sangunaria canadensis, which contains a large amount of 1, has now been added into toothpastes in USA. Macleaya cordata also contains other alkaloids of protopine type, protopine 3 and allocryptopine 4, which have not been reported to have strong

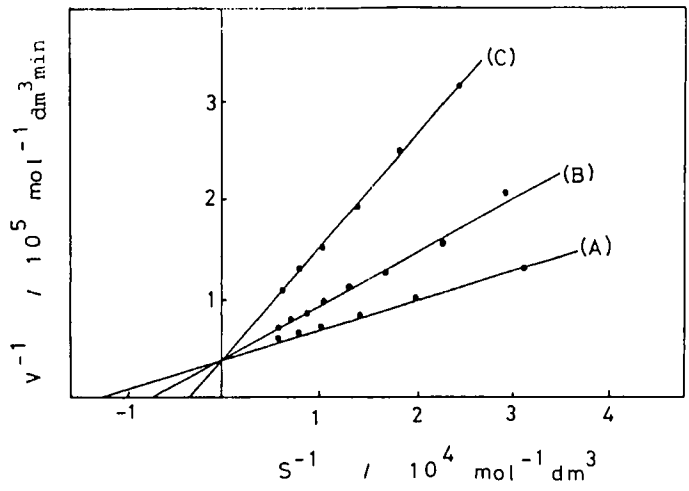


Fig. 6 1/S - 1/V plots of papaverine
(A) blank, (B) 20 μM (C) 50 μM

bactericidal activity. Since inhibitory action of these alkaloids was not as yet investigated, we examined their effects on PDE activity. As depicted in Fig. 7, it was found out that both 1 and 2 competitively inhibit PDE similar to 8. The inhibition by 1 somewhat stronger than 2. On the contrary, 3 and 4 did not exhibit remarkable inhibition (< 10 %) even when the concentration of these alkaoids was set to be equal to the initial concentration of CAMP. The present results suggest that the inhibition of PDE closely related to bacetricidal activity for these alkaloids. The inhibition by a few related compounds was also examined. The reduction product of 1 and 2 (6 and 7, respectively) was found to inhibit PDE activity competitively, the inhibition being weaker than 1 and 2. The inhibition of clelidonin 5, the major alkaloid of Chelidonium majus and belongs to hydrated derivatives of benzo[c]phenanthridine alkaloids was also examined. It was found

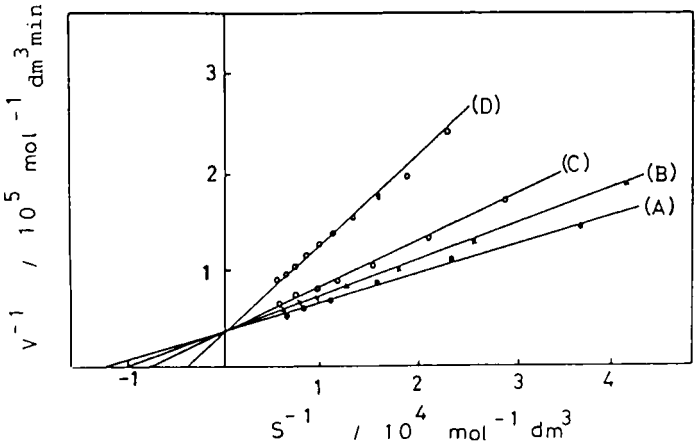


Fig. 7 1/S - 1/V plots of benzo[c]phenanthridine alkaloids
(A) blank, (B) chelerythrine 20 μM, (C) sanguinarine 20 μM, (D) sanguinarine 50 μM

Table 2 Inhibition constants of benzo[c]phenanthridine and related alkaloids

Compounds	Name	Inhibition constants (K_i / M)
1	sanguinarine	4×10^{-5}
2	chelerythrine	8×10^{-5}
3	protopine	$> 10^{-3}$
4	allocryptopine	$> 10^{-3}$
5	(+)-chelidonine	4×10^{-4}
6	dihydrosabguinarine	2×10^{-4}
7	dihydrochelerythrine	2×10^{-4}
8	papaverine	2×10^{-5}

that 5 also competitively inhibit PDE activity and its inhibition action was weaker than 1 and 2. Table 2 summarizes the inhibition constants of the alkaloids tested in the present study. Since each measuremnts finishes within 20 to 40 min, determination of inhibition constant and elucidation of the mode of inhibition was achieved only within 1 to 2 hr for each substance to be tested. The present method will be useful when the reaction proceeds at a speed suitable for LC assay and the time required for a single LC separation is short enough. The rate of reaction can be controlled to some extent by the choice of the initial concentration of the substrate and the amount of enzyme added. The time required for a single LC separation can be reduced if fast-LC column is used instead of conventional one. Repeated injection of macromolecular enzyme will inevitably induce the degradation of the column, but the degradation may be minimized by the proper use of precolumn filter or precolumn. When these conditions are fulfilled and the sample volume can be chosen not so small (at least a few mililitter), the present method may be a useful approach for the assay of enzyme reactions.

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