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Simultaneous Determination of Multiple Enzyme Activities by High Performance Liquid Chromatography

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A method is proposed for the simultaneous determination of multiple enzyme activities in a specimen by high performance liquid chromatography. To examine the applicability of the method, the hydrolytic activities of arylsulfatase, β-glucosidase and β-glucuronidase, all having broad optimal pH ranges around 5 were assayed by quantitative determination of 8-hydroxyquinoline (oxine, Ox), 5,7-dichloro-8-hydroxyquinoline (Di-Cl) and 5-chloro-7-iodo-8-hydroxyquinoline (chinoform, CF), the products released enzymatically from Ox-β-D-glucoside, Di-Cl-sulfate and CF-β-D-glucuronide. The method was applied to rat organs and *Bifidobacterium infantis* No. 9.

Keywords——high performance liquid chromatography; β-glucosidase; arylsulfatase; β-glucuronidase; 8-hydroxyquinolines; enzyme assay; UV detection; rat organs; bacterial enzymes

Determination of enzyme activities in a small volume of sample is important in many areas of biochemistry and clinical chemistry. For this purpose much work has been carried out to increase the sensitivity and to simplify the procedures. Developments in instrumentation have allowed multiple enzyme activities to be assayed by multi-channel automated systems.[2] The principle of such systems has been based on the separate determination of each enzyme activity. However, if assay of multiple enzyme activities in a specimen can be achieved by a simultaneous determination of the products released after incubation with multiple substrates, this would be more convenient and would facilitate pattern analysis.

In the present investigation, activities of β-glucosidase, arylsulfatase and β-glucuronidase, all having an optimal pH around 5, were determined simultaneously by high performance liquid chromatography (HPLC), using 8-hydroxyquinoline (oxine, Ox)-β-D-glucoside (Ox-glucoside), 5,7-dichloro-8-hydroxyquinoline(Di-Cl)-sulfate(Di-Cl-sulfate) and 5-chloro-7-iodo-8-hydroxyquinoline(chinoform, CF)-β-D-glucuronide(CF-glucuronide) as substrates.

Experimental

Materials——Ox was purchased from Tokyo Kasei (Tokyo, Japan). 5-Chloro-8-hydroxyquinoline (5-Cl), Di-Cl and CF, kindly provided by Tanabe Seiyaku (Tokyo, Japan), were purified by recrystallization.[3] Ox-glucoside was purchased from Sigma (St. Louis, Mo., U.S.A.). Di-Cl-sulfate was synthesized according to the method of Chen *et al.*[4] CF-glucuronide was synthesized as described earlier.[5]

β-Glucosidase, β-glucuronidase (Worthington; Freehold, N.J., U.S.A.), and arylsulfatase (type II from limpets, Sigma) were used without further purification. These enzymes were dissolved in aqueous 0.01% bovine serum albumin[6] and used as authentic enzyme solutions.

All the other reagents used were of analytical grade.

Preparation of Enzyme Specimens——Female rats (Donryu), weighing 200—250 g were killed by decapitation. The livers and kidneys were rapidly removed, homogenized in a Potter-Elvehjem glass homogenizer

1) Location: *7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan.*

2) A.A. Tappel, "Methods in Enzymology," Vol. 22, ed. by W.B. Jakoby, Academic Press, New York, 1969, p. 14.
3) C.T. Chen, K. Samejima, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 97 (1976).
4) C.T. Chen, K. Samejima, and Z. Tamura, *Igaku no Ayumi* (Japan), **84**, 195 (1973).
5) I. Matsunaga and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 1056 (1971).
6) P. Bernfeld, H.C. Bernfeld, J.S. Nisselbaum, and W.H. Fishman, *J. Am. Chem. Soc.*, **76**, 4872 (1954).

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by adding distilled water to make a 50 mg/ml mixture (w/v), and centrifuged at $1200 \times g$ for 5 min. The supernatant was diluted with distilled water to an appropriate dilution. All the procedures were performed at 4°.

Bifidobacterium infantis No. 9 was cultured in Negishi selective medium[7] under CO_2–N_2 (1:9) at 37° for one day. Cells were harvested by centrifugation and washed three times with physiological saline supplemented with 0.05% cysteine. The cell suspension obtained was ultrasonicated in an ice-water bath using a Sonic 300 Dismembrator (Artek Systems Corp., Fermingdale, N.Y., U.S.A.) with a standard tip (13 mm ϕ) at 70% of full power for 20 min. The ultrasonicated suspension was used directly as an enzyme specimen.

Incubation——The enzyme substrates were dissolved in distilled water to make stock solutions at room temperature (20°). Thus concentrations of the stock solutions were 10 mM, 20 mM and 5 mM for Ox-glucoside, Di-Cl-sulfate and CF-glucuronide, respectively. Acetate buffer solution (final concentration, 0.1 M)[8] and the stock solutions of these substrates (final concentration, 1 mM each) were added to the enzyme specimen and the mixture was incubated at 37° for one hr.

HPLC——The liquid chromatograph consisted of a minimicro pump (type KSU-45H, Kyowa Seimitsu, Tokyo), a column of IATROBEADS 6CP-2010 resin (10 μm, polystyrene-type porous polymer, Iatron Labs., Tokyo) packed in a glass tube 3 mm × 18 cm, and a low pressure mercury vapor lamp (254 nm). The temperature of the column was maintained at 50±0.5° by a thermostated water bath with a water circulating apparatus (BT-35, Yamato Scientific, Tokyo).

Quantification——The enzyme activity of tissue was measured in terms of the amount of each 8-hydroxyquinoline produced from the substrate at 37° in one hr per mg of wet tissue. The blank was performed with a heat-denatured specimen (5 min in a boiling water bath). Each 8-hydroxyquinoline was determined by using the relationship between the peak height ratio relative to the internal standard and the amount of the 8-hydroxyquinoline. In the usual measurements, 80 nmol of 5-Cl was added to the incubation mixture after the enzymatic reaction as an internal standard.

Results

Separation of 8-Hydroxyquinolines by HPLC

In view of the known properties of the 8-hydroxyquinolines, a combination of a nonpolar resin with a mixture of alcohol and acidic buffer was expected to give good resolution. Of several resins examined, a polystyrene-type porous polymer (IATROBEADS 6CP-2010) gave the highest resolution when combined with a mixture of 0.1 M glycine–hydrochloric acid (pH 3.0) and methanol (10:90, v/v) at 50°. As shown in Fig. 1, addition of 2% *n*-hexane to the eluent reduced the retention time without affecting the resolution. Determination of authentic 8-hydroxyquinolines showed good reproducibility with the HPLC system (Table I).

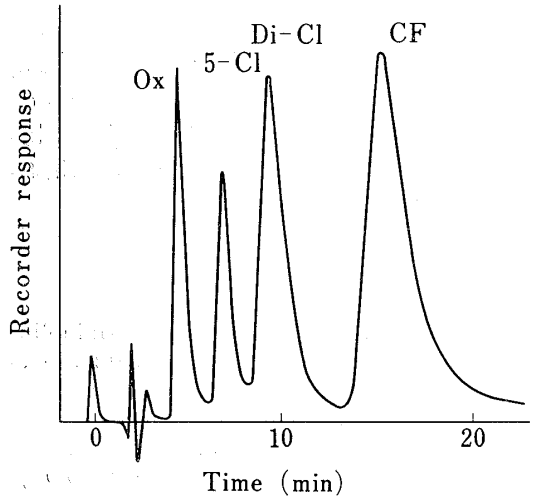

Fig. 1. HPLC Separation of 8-Hydroxyquinolines

Column: IATROBEADS 6CP-2010 (10 μm, 3 mmϕ × 18 cm, 50°).
Eluent: 0.1 M glycine-HCl, pH 3.0/methanol/*n*-hexane=10:88:2 (by volume).
Flow rate: 0.50 ml/min.
Detector: UV (254 nm); range 0.04 A.U.F.S.
Sample amount: 1 nmol each in methanol.

Table I. Reproducibility of the HPLC Procedure by UV Detection

	Peak height ratio		
	Ox/5-Cl	Di-Cl/5-Cl	CF/5-Cl
Mean ($N=10$)	1.38	1.28	1.17
σ_{N-1}	0.021	0.049	0.036
C.V. (%)	1.5	3.8	3.1

A methanolic solution containing one nmol of 8-hydroxyquinolines was injected into the HPLC system under the conditions described in Fig. 1.

7) M. Yoshioka, S. Yoshioka, Z. Tamura, and K. Ohta, *Japan. J. Microbiol.*, 12, 395 (1968).
8) D. W. Bradley and A.L. Tappel, *Anal. Biochem.*, 33, 400 (1970).

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Extraction of the Products

Although the enzyme substrates have intense absorbance around 254 nm, like the products (Table II), the former were eluted at the solvent front and thus did not interfere with the assay. When the enzymatic reaction mixture was injected directly to the HPLC system, Ox and 5-Cl were detected but Di-Cl and CF were not detected owing to their strong adsorption on proteins. This confirmed the need for the extraction procedure. Chen *et al.*[3] used pyridine–benzene (1: 9) for the extraction of CF from serum. This solvent system was also suitable for the extraction of Ox, 5-Cl and Di-Cl as well as CF from rat liver homogenate. During evaporation of the pyridine–benzene (1: 9) extract, Ox was found to sublime. However, the loss of Ox was completely prevented by adding NaOH to the solution.

Table II. UV Absorption Maxima and Molar Extinction Coefficients of the Substrates, Products and the Internal Standard

	λ_{max} (nm)	log ε
Ox-β-D-glucoside	236	4.51
Ox	242	4.52
Di-Cl-sulfate	238	4.48
Di-Cl	248	4.59
CF-β-D-glucuronide	245	4.48
CF	255	4.63
5-Cl	246	4.48

UV absorbance was measured with 10 μM aqueous (substrates) or methanolic (products and internal standards) solutions using a Shimadzu spectrophotometer (type UV-202).

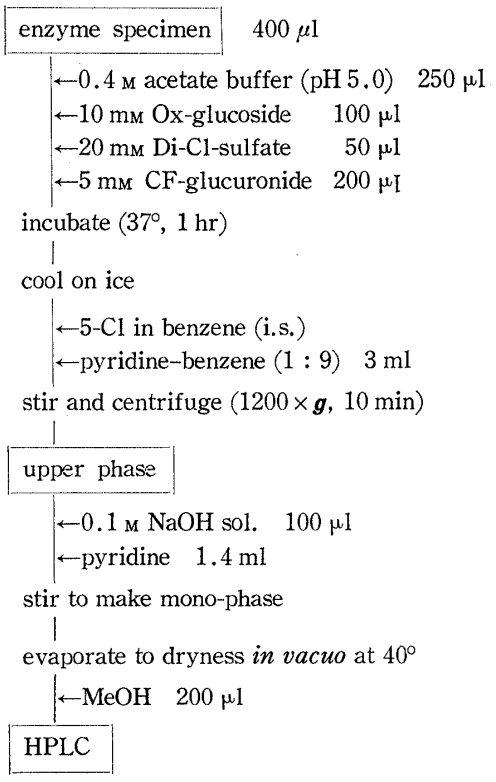

Chart 1. Recommended Procedures for the Assay of Multiple Enzyme Activities by HPLC

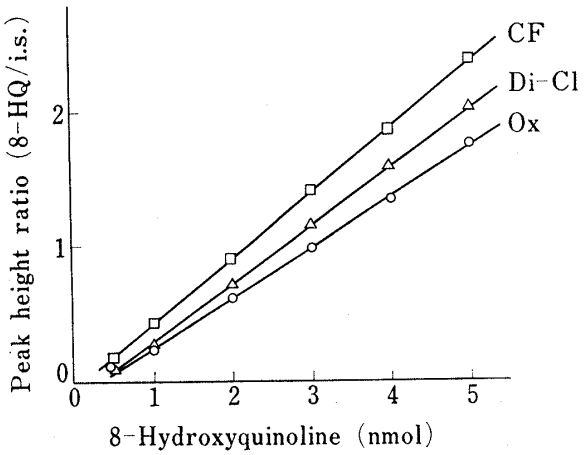

Fig. 2. Working Curves for 8-Hydroxyquinolines

A mixture of Ox, Di-Cl and CF in benzene (1 mM each) was prepared and 10, 20, 40, 60, 80 and 100 μl aliquots were taken into tubes. Then, 80 μl of 1 mM 5-Cl solution (i.s.) in benzene, 0.5 ml of pyridine and 100 μl of 0.1 M NaOH were added in succession. The mixture was evaporated to dryness *in vacuo* by aspiration at 40°, 200 μl of methanol was added, and an aliquot (10 μl) was injected into the HPLC system. Each point is the mean of five samples.

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Established Procedures

The total procedure is shown in Chart 1. Figure 2 shows working curves for 8-hydroxyquinolines. The lower limit of determination was approximately 0.5 nmol of 8-hydroxyquinoline. Table III shows the recoveries obtained by the standard-addition method using ten-fold diluted rat liver homogenate. Because of the high affinity of 8-hydroxyquinolines for proteins and lipids, their recovery was incomplete, especially when small quantities of compounds were employed.

Table III. Recovery of 8-Hydroxyquinolines from Rat Liver Homogenate

Compound	Added (nmol)	Recovered[a] (nmol) (Mean±S.E.)
Ox	10	8.4±0.11
	100	91 ±3.0
Di-Cl	10	7.6±0.61
	100	95 ±1.8
CF	10	7.4±0.75
	100	95 ±1.9

a) Mean of three samples.

Ten-fold diluted supernatant of rat liver homogenate (50 mg/ml) was used. Taking into account the poor solubility of 8-hydroxyquinolines at pH 5.0, the following procedure was employed.

A benzene solution of Ox, Di-Cl and CF (10 nmol or 100 nmol each) was mixed with 100 μl of 0.1 M NaOH and 0.5 ml of pyridine and evaporated to dryness. To the residue, 100 μl of distilled water, 0.4 ml of rat liver homogenate and 0.5 ml of 0.2 M acetate buffer (pH 5.0) were added and the mixture was incubated at 37° for one hr. After incubation, internal standard was added (5-Cl, 8 nmol or 80 nmol for each range) together with 3 ml pyridine–benzene (1: 9) for extraction. The mixture was stirred and centrifuged at 1200×*g* for 10 min and the upper phase was transferred to another tube. The extract was mixed with 100 μl of 0.1 M NaOH and 1.4 ml of pyridine, evaporated to dryness, and 200 μl of methanol was added. An aliquot was injected into the chromatograph.

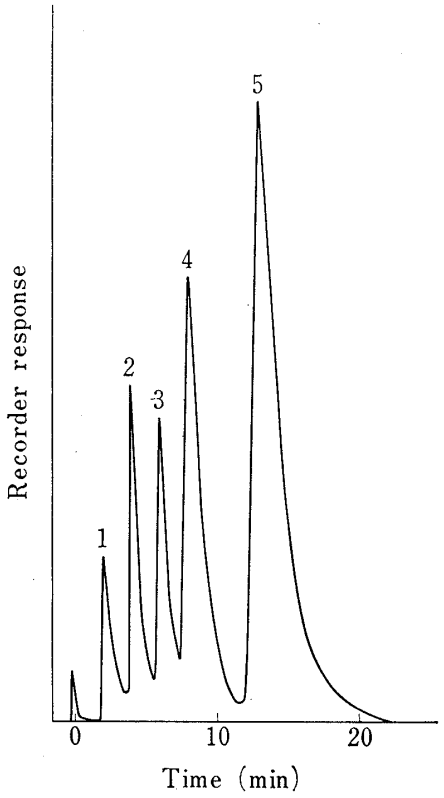

Fig. 3. Multiple Enzyme Activity Determination of Authentic Enzyme Mixture

As an enzyme mixture, 0.02 mg of β-glucosidase, 0.2 mg of arylsulfatase and 0.006 mg of β-glucuronidase were mixed with 80 nmol of 5-Cl (internal standard) in a tube and treated by the procedure shown in Chart 1. Peak identity: 1, solvent front; 2, β-glucosidase activity; 3, internal standard; 4, arylsulfatase activity; 5, β-glucuronidase activity.

Application of the Method

When the established procedure was used for the assay of the three enzymes, the detection limits were 2 μg of the β-glucosidase, 20 μg of the arylsulfatase and 1 μg of the β-glucuronidase. A typical chromatogram of multiple enzyme determination from mixed authentic enzymes is shown in Fig. 3.

When the present method was applied to rat organs to obtain rough estimates of the enzyme activities without correction for the recovery of the products, arylsulfatase and β-glucuronidase activities were detected in the liver homogenate, which released 40 nmol of Di-Cl and 1.1 μmol of CF per mg of tissue in one hr. Much lower activities of arylsulfatase and β-glucuronidase were detected in rat kidney homogenate, which produced 5.7 nmol of Di-Cl and 25 nmol of CF per mg of tissue in one hr. β-Glucosidase activity in terms of a

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peak of Ox was not detected in an ultrasonicated solution of *Bifidobacterium infantis* No. 9. Some typical chromatograms are shown in Fig. 4.

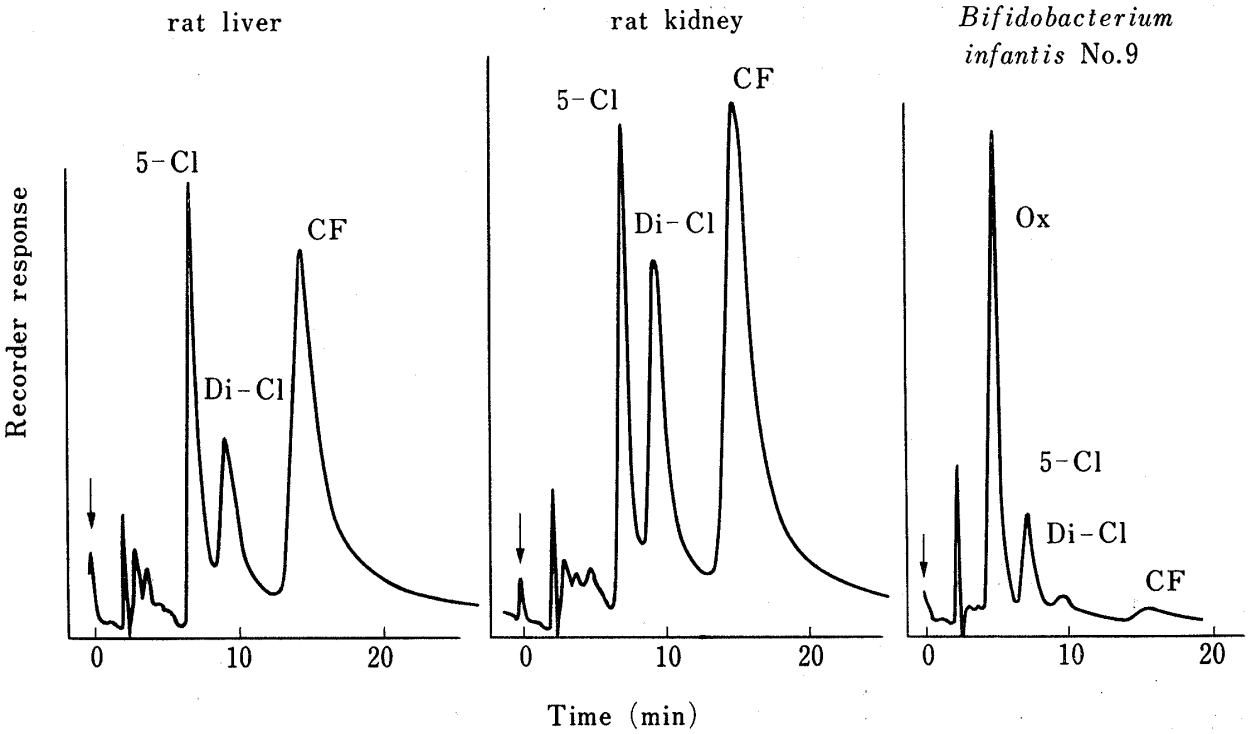

Fig. 4. Multiple Enzyme Activities detected in Various Enzyme Specimens

Discussion

Simultaneous determination of multiple enzyme activities by HPLC was shown to be possible in the present investigation. Use of 8-hydroxyquinoline derivatives as substrates had several advantages. They have large molar extinction coefficients and similar absorption maxima, which permitted the sensitive determination of 8-hydroxyquinolines at similar levels. On the other hand, high affinity of 8-hydroxyquinolines for high-molecularweight biological substances required the extraction of the products, which made the whole procedure complicated and time-consuming. Loss of Ox and 5-Cl by sublimation was avoided by the addition of NaOH. The substrates, especially Di–Cl-sulfate and CF-glucuronide, are known to be non-enzymatically hydrolyzed in the presence of some metal ions.[9] Thus, a blank is always required for accurate determination of the enzyme activities.

A similar method can be applied to other hydrolytic enzymes such as glycosidases, esterases and lipases, and also to various transferases. In such cases, the optimal pH values should be nearly the same, and the incubation buffer should be carefully selected to avoid inhibition of the enzymes. Use of fluorogenic substrates should improve the method.

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