[Chem. Pharm. Bull. 31(5)1666—1669(1983)]

Assay for Pyrimidine Nucleoside Phosphorylases in Tissue Extracts by High-Performance Liquid Chromatography

Akira Kono,*,[a] Setsuro Sugata,[a] Yasuhiro Hara,[a] Mutsuko Tanaka,[a] Yoshiharu Karube,[a] and Yoshikazu Matsushima[b]

Kyushu Cancer Center Research Institute,[a] Notame, Minami-ku, Fukuoka 815, Japan and Kyoritsu College of Pharmacy,[b] Shibakoen 1-5-30, Minato-ku, Tokyo 105, Japan

(Received October 18, 1982)

A sensitive method for the assay of pyrimidine nucleoside phosphorylases in preparations of human and animal tissues by determination of pyrimidines is described. Pyrimidines formed enzymatically from thymidine, uridine, 5-fluorouridine, and 5′-deoxy-5-fluorouridine are determined by means of high-performance liquid chromatography with ultraviolet (UV) detection. The pyrimidines, after extraction with ethyl acetate, are separated by reversed-phase chromatography on μ-Bondapak C-18/Porasil. The limits of detection are 2.5, 1.0, and 2.0 pmol for thymine, uracil, and 5-fluorouracil, respectively.

Keywords——thymidine phosphorylase; uridine phosphorylase; thymidine; thymine; uridine; uracil; 5-fluorouracil; 5-fluorouridine; 5′-deoxy-5-fluorouridine; 5-chlorouracil; HPLC.

Pyrimidine nucleoside phosphorylases play important roles in the metabolism of nucleic acids and are present in normal and neoplastic tissues. It has been claimed that they are responsible for the catabolism of chemical depot forms of 5-fluorouracil (FU) such as 5′-deoxy-5-fluorouridine (DFUR) and 1-(tetrahydro-2-furanyl)-5-fluorouracil (Tegafur) with real or potential chemotherapeutic usefulness.[1–6]

There are two distinct pyrimidine nucleoside phosphorylases. One is thymidine phosphorylase (EC 2.4.2.4), which catalyzes the reversible conversion of thymidine (dThd) and phosphate to thymine and 2-deoxyribose-1-phosphate. The other phosphorylase is uridine phosphorylase (EC 2.4.2.3), which catalyzes the reversible conversion of uridine (Urd) and phosphate to uracil and ribose-1-phosphate. Recently, the existence of a third pyrimidine nucleoside phosphorylase activity, uridine-deoxyuridine phosphorylase, was suggested.[6]

Several assay methods have been proposed for pyrimidine nucleoside phosphorylases. Some are based on the differences in the ultraviolet (UV) spectra of nucleosides and pyrimidine base in strong alkaline solutions.[7–9] In the continuous spectrometric methods, the change in absorbance which accompanies conversion of nucleosides to bases was monitored.[10,11] Most of the assay methods are based on the separation and determination of the phosphorolyzed products, either sugar[12,13] or base moieties. Radiolabeled substrates are frequently used in the determination of the bases.[3–6,14] Enzymatic activity for phosphorolysis of DFUR can be measured by the bacterial method.[15] Generally, spectrometric methods suffer from inaccuracies and the separation methods are tedious.

High-performance liquid chromatography (HPLC) has become an ordinary laboratory technique, and is a simple and accurate method for the separation and determination of a large number of compounds. HPLC was successfully applied for the determination of pyrimidines in incubation mixtures of nucleosides and FU derivatives with tissue extracts.[1–3] The present paper describes a procedure for the assay of pyrimidine nucleoside phosphorylases based on the determination of pyrimidines by HPLC.

Experimental

Materials——All chemicals were of reagent grade. Deionized and distilled water was used. DFUR was provided by Hoffmann-La Roche Inc., Nutley, N.J., U.S.A. 1-(2′-Deoxy-β-D-glucopyranosyl)thymine

(GPT) (NSC 402666) was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, U.S.A.

Enzyme preparations were obtained from tumor and normal tissues of humans and of experimental animals. Tissues were homogenized and centrifuged at 105000×*g* for 90 min at 4°C. Each supernatant was dialyzed against 500 volumes of 20 mM phosphate buffer (pH, 7.4). The clear solution thus obtained was used as an enzyme preparation. The preparation was further purified through fractional precipitation and chromatography. Solutions obtained in various stages of purification procedures were also used as enzyme preparations.

The protein concentrations in the enzyme preparations were determined by the method of Lowry *et al.*[16] using bovine serum albumin as a standard.

Assay Procedure——In a 20-ml test tube, 0.2 ml of aqueous solution of pyrimidine nucleoside, 0.2 ml of 0.36 M phosphate buffer (pH, 7.4), 0.2 ml of 0.49 M NaCl, and 0.2 ml of 12 mM KCl were mixed. The mixture was preincubated at 37°C for 5 min, then incubated again at 37°C after addition of 0.2 ml of enzyme preparation. Concentrations of pyrimidine nucleosides were varied in the range of 16 μM to 10 mM and the incuba tion time was varied from 5—60 min according to the enzyme activity of the preparations. The reaction was terminated by addition of 8 ml of cold ethyl acetate. Then 0.1 ml of aqueous solution of an internal standard and 0.1 ml of 0.5 M NaH_2PO_4 were added. The mixture was shaken vigorously for 10 min and centrifuged (3000 rpm, 10 min). The organic layer was separated and concentrated *in vacuo*. The residue was dissolved in methanol. A portion of the methanol solution was injected into the liquid chromatograph.

HPLC Instrumentation——A Waters model 204 liquid chromatograph equipped with a model 6000 solvent delivery system, a model U6K universal injector, and a model 440 UV detector operated at 254 nm was used. The column was μ-Bondapak C-18/Porasil (particle size, 8—10 μm; 300×3.9 mm i.d.). The mobile phase was water, and its flow rate was 1 ml/min at a pressure of about 1100 p.s.i.

Results and Discussion

A single chromatographic peak was observed for a pyrimidine when a methanol solution of the pyrimidine was injected into the chromatograph. When an aqueous solution of the pyrimidine was taken through the assay procedure instead of a nucleoside, the same chromatographic peak was obtained. The retention times were 5.5 min for uracil, 6.0 min for FU, 9.0 min for 5-chlorouracil (CU), and 10.5 min for thymine.

Similarly, pyrimidine nucleosides each gave a single peak. The retention times of nucleosides were 8.7 min for Urd, 10.7 min for 5-fluorouridine (FUR), 13.8 min for GPT, 24.5 min for dThd, and 25.5 min for DFUR. In the present study, dThd, Urd, DFUR, and FUR were used as substrates. GPT is a specific inhibitor of uridine phosphorylase, but does not inhibit the activity of thymidine phosphorylase. GPT did not interfere with the measurement of the pyrimidines. Inhibition studies with GPT can give an indication of the type of enzyme responsible for the phosphorolysis of a pyrimidine nucleoside.[1,2,6]

Because of its convenient retention time, CU was used as an internal standard (IS) for the measurements of thymine and FU. For the measurement of uracil, thymine was used as the IS.

Standard solutions containing known amounts of the pyrimidines were carried through the assay procedure. Calibration curves obtained by plotting the ratio of peak height of the pyrimidines to that of the IS against the amount of pyrimidine were linear up to 16 nmol and passed through the origin. The limits of detection were 2.5, 1.0, and 2.0 pmol for thymine, uracil, and FU, respectively. The precision was established with respect to repeatability. The coefficient of variation was 2.9% for a mean value of 6.40 nmol thymine ($n=7$).

The conditions for the enzyme reaction described in the experimental procedure are optimal. Typical chromatograms obtained according to the procedure are shown in Fig. 1. The enzyme preparation used was obtained from a human gastric cancer (poorly differentiated adenocarcinoma from a 58-year-old female). Substrates were dThd, Urd, and DFUR for Fig. 1 (a), (b), and (c), respectively. Peaks of the substrate, product, and the internal standard were well separated. No interfering peak arose from components of the enzyme preparations. Results are summarized in Table I. The activities of the enzymes are expressed in terms of the amount of the pyrimidines produced (nmol) in 1 h by 1 mg protein.

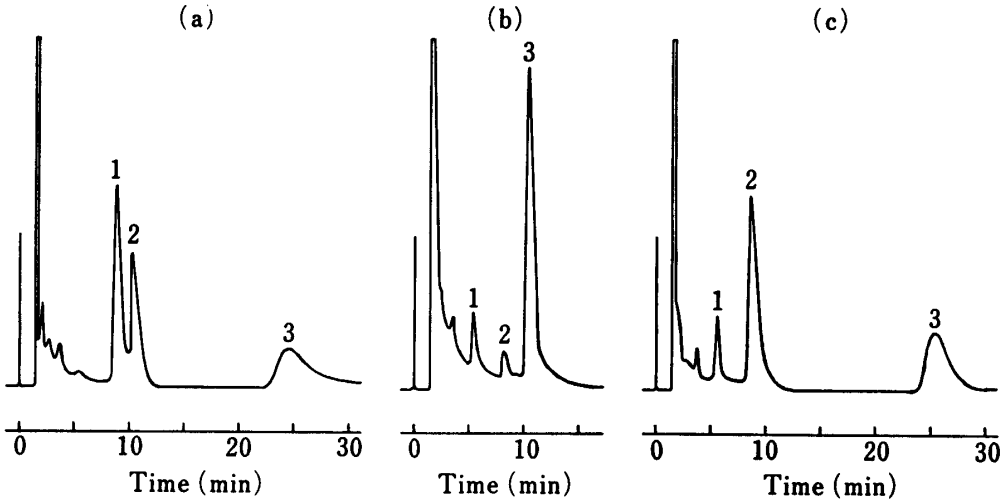

Fig. 1. Chromatograms obtained according to the Standard Procedure

Substrates are (a) dThd, (b) Urd, and (c) DFUR. Peaks: (a) 1=CU (IS); 2=thymine; 3=dThd. (b) 1=uracil; 2=Urd; 3=thymine (IS). (c) 1=FU; 2=CU (IS); 3=DFUR.

TABLE I

Substrate[a)]	Incubation time (min)	Pyrimidine (nmol)	Protein content (μg)	Enzyme activity (nmol·h^{-1}·mg^{-1})
(a) dThd	30	6.40	87	147
(b) Urd	45	2.07	174	15.9
(c) DFUR	45	2.10	174	16.1

a) Concentrations of substrates; 16 μM.

Negligible amounts of the pyrimidines were formed in the absence of enzyme preparations or by the use of heat-treated (100°C, 15 min) enzyme preparations. The pyrimidines were not formed in significant amounts in the absence of inorganic phosphate (in Tris buffer). The results indicate that the pyrimidines are formed enzymatically by the phosphorolytic cleavage of the nucleosides.

The HPLC method is sensitive and precise. Kinetic studies on pyrimidine nucleoside phosphorylases in normal and tumor tissues of human organs are in progress using the present method, and the results will be reported, shortly.

References and Notes

1) A. Kono, Y. Hara, and Y. Matsushima, *Chem. Pharm. Bull.*, **29**, 1486 (1981).
2) A. Kono, Y. Hara, S. Sugata, Y. Karube, Y. Matsushima, and H. Ishitsuka, *Chem. Pharm. Bull.*, **31**, 175 (1983).
3) T. Shirasaka, S. Nagayama, S. Kitano, Y. Maehara, and S. Fujii, *Gan-to-Kagakuryoho*, **8**, 262 (1981).
4) H. Ishitsuka, M. Miwa, K. Takemoto, K. Fukuoka, A. Itoga, and H.B. Maruyama, *Gann*, **71**, 112 (1980).
5) G.D. Birnie, H. Kroeger, and C. Heidelberger, *Biochemistry*, **2**, 566 (1963).
6) P.W. Woodman, A.M. Sarrif, and C. Heidelberger, *Cancer Res.*, **40**, 507 (1980).
7) E.W. Yamada, *Can. J. Biochem.*, **42**, 317 (1964); *Idem*, *Biochim. Biophys. Acta*, **149**, 299 (1967); *idem*, *J. Biol. Chem.*, **243**, 1649 (1968); K. Kraut and E.W. Yamada, *J. Biol. Chem.*, **246**, 2021 (1971).
8) M. Friedkin and D. Roberts, *J. Biol. Chem.*, **243**, 4936 (1968).
9) M. Schwartz, *Methods Enzymol.*, **51**, 441 (1978).
10) T.A. Krenitsky, *Biochim. Biophys. Acta*, **429**, 352 (1976).
11) C. Nakayama, Y. Wataya, R.B. Meyer, Jr., D.V. Santi, M. Saneyoshi, and T. Ueda, *J. Med. Chem.*, **23**, 962 (1980).

12) H. Pontis, G. Degerstedt, and P. Reichard, *Biochim. Biophys. Acta*, **51**, 138 (1961).
13) M. Zimmermann and J. Seidenberg, *J. Biol. Chem.*, **239**, 2618 (1964).
14) Y. Maehara, H. Nakamura, Y. Nakane, K. Kawai, M. Okamoto, S. Nagayama, T. Shirasaka, and S. Fujii, *Gann*, **73**, 289 (1982).
15) M. Miwa, J. Nakamura, and H. Ishitsuka, *Gann*, **72**, 965 (1981).
16) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).