Journal of Chromatography, 356 (1986) 445–449
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 373

Note

Determination of 5-fluorouridine diphosphate glucose as a metabolite of 5-fluorouracil in mouse T-lymphoma (S-49) cells using high-performance liquid chromatography

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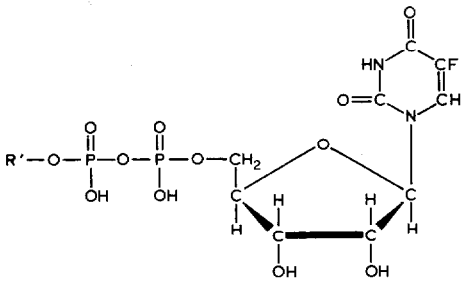
(Received November 25th, 1985)

Recently a high-performance liquid chromatography (HPLC) procedure capable of resolving all of the potential intracellular metabolites of 5-fluorouracil (5-FUra) was reported[1]. The authors indicated that the 5-fluorouridine diphosphate (FUDP)-hexose metabolites of 5-FUra have not received serious consideration as important metabolites because previous methodology was not sufficient for their separation.



FUDP R′ = H
FUDP-Glucose R′ = Glucose
FUDP-Galactose R′ = Galactose

Previously, a HPLC assay procedure for the complete analysis of the pyrimidine ribo- and deoxyribonucleotide pools in tissue samples was reported from this laboratory and its applicability was demonstrated in cultured cells following exposure to 5-FUra[2,3]. This report describes a procedure for the determination of two FUDP-hexoses as metabolites of 5-FUra following prior separation by HPLC. With this additional procedure, the HPLC method[2] has the advantage of permitting not only an observation of the pool-size changes of the natural pyrimidine nucleotides but also analysis of the major intracellular metabolites of 5-FUra.

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0021-9673/86/$03.50

MATERIALS AND METHODS

Reagents

Nucleotide and nucleoside standard samples, and all other reagents, were of analytical grade. Aquasol was obtained from New England Nuclear (Boston, MA, U.S.A.); 5-fluoro[6-^{3}H]uracil (15–20 Ci/mmol) was from Moravek Biochemicals (City of Industry, CA, U.S.A.); alkaline phosphatase (E.C. 3.1.3.1) suspension, uridine-5′-diphosphoglucose (UDPG) pyrophosphorylase (E.C. 2.7.7.9) as a lyophilized powder, pyrophosphatase nucleotide (E.C. 3.6.1.9) Type III as a lyophilized powder and inorganic pyrophosphatase (E.C. 3.6.1.1.) as a lyophilized powder were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Standard solutions

The internal standard for the Aminex A-29 column was a solution of adenosine triphosphate (ATP) at a concentration of 1 nmol/μl in distilled water. The internal standard for the preparative column was a solution of 5-fluorouridine (FUR) and 5-fluorodeoxyuridine (FdUR) each at a concentration of 2 mg/ml in distilled water. The pyrophosphatase nucleotide solution was prepared by dissolving 1.9 mg of the enzyme preparation in 1.25 ml of distilled water.

Chromatography

HPLC analysis was performed on a liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) equipped with a Model U6K injector, a Model 440 dual wavelength (254/280 nm) UV absorbance detector, a Model 660 solvent programmer and two Model M6000-A pumps. Columns and conditions used were: (a) Aminex A-29 anion-exchange column with gradient elution as previously described[2] and (b) preparative μBondapak C_{18} reversed-phase column, 30 cm × 7.8 mm I.D., average particle diameter 10 μm (Waters Assoc.); mobile phase, acetonitrile–water (1.0:99.0), filtered and deaerated (0.45 μm; Millipore, Bedford, MA, U.S.A.), flow-rate 6.0 ml/min; temperature, ambient; recorder chart speed, 1 cm/min.

Cell culture preparation

Wild Type S-49 mouse T-lymphoma cells were grown in suspension in Dulbecco's modified Eagle's medium supplemented with 10% horse serum[4,5]. Cells were incubated in a total volume of 10 ml culture medium (initial density 10^5 cells/ml for 24 h at 37°C in the presence of 0.9 μM [6-^{3}H]FUra. The culture medium was then treated as previously described to provide a sample for HPLC analysis[2].

Detection of FUra metabolites

The sample prepared from the cell culture was injected onto the Aminex A-29 anion-exchange column and eluted using a gradient system[2]. Fractions containing 0.9 ml were collected at 3-min intervals using a Model 2112 Redirac fraction collector (LKB, Stockholm, Sweden). A 100-μl aliquot of each fraction was placed in a scintillation vial, 10 ml of Aquasol were added and the tritium content determined using a LS-9000 liquid scintillation counter (Beckman, Berkeley, CA, U.S.A.). A radioactivity profile of the chromatogram is shown in Fig. 1. Fractions 44–62 from the Fig. 1 chromatogram were used to determine the presence of FUDP-hexoses.

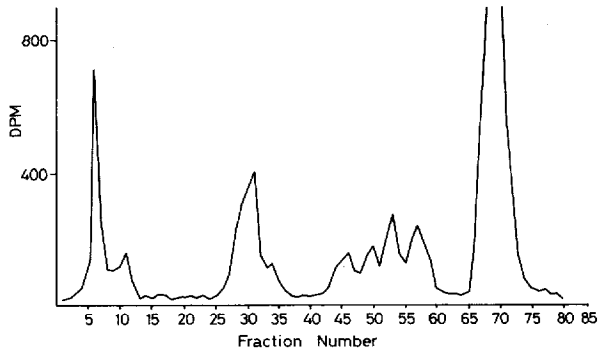
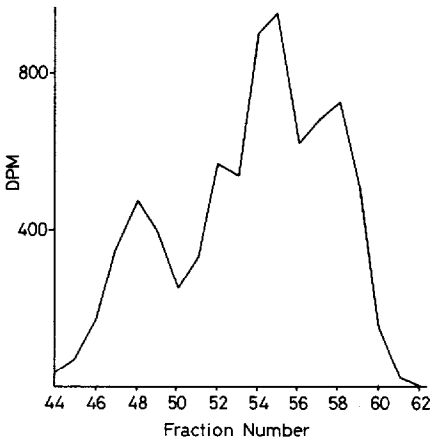


Fig. 1. Aminex A-29 radioactivity chromatogram of cell extract prepared after treating S-49 cells with [6-^{3}H]FUra.

Determination of 5-FUra-nucleotides

Fractions 44–62 were each treated with alkaline phosphatase to determine the contribution of 5-FUra-nucleotides to total radioactivity of each sample as follows: into a 1.5-ml snap-cap polypropylene centrifuge-tube were placed 250 μl of the fraction to be analyzed, 3 μl of alkaline phosphatase and 25 μl of 0.01 *M* sodium hydroxide solution. The contents of the tube were mixed and then heated at 37°C for 40 min. After heating, 25 μl of the internal standard solution (FUR + FdUR) and 25 μl of 50% acetic acid were added. The contents were mixed, a 50-μl portion was removed and placed in a scintillation vial along with 10 ml Aquasol and the radioactivity content determined in order to serve as a control as shown in Fig. 2. Each phosphatase treated sample was chromatographed using the preparative C_{18} column and the fractions corresponding to solvent front, FUR and FdUR as shown in Fig. 3 were collected in scintillation vials. The fractions from the preparative column were concentrated to 1 ml on a hot plate under a stream of nitrogen, not exceeding a



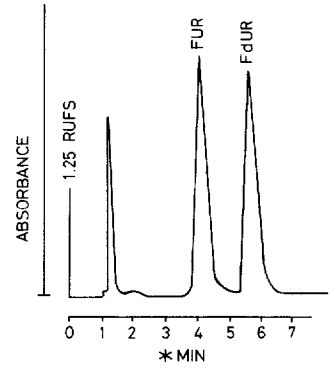


Fig. 2. Radioactivity profile of fractions 44–62 after treatment with alkaline phosphatase.

Fig. 3. Preparative reversed-phase chromatogram of FUR and FdUR.

temperature of 65°C and the radioactivity then determined after the addition of 10 ml quasol. The radioactivity profile for the phosphatase treated samples after chromatography is shown in Fig. 4.

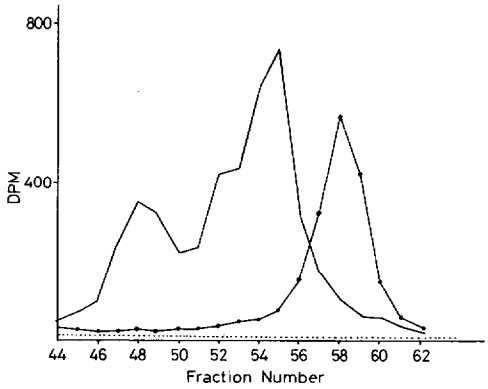
Determination of FUDP-hexoses

Fractions 44–62 were each treated with pyrophosphatase plus alkaline phosphatase to determine the contribution of 5-fluorouridine-diphosphohexose to each sample as follows: into a 1.5-ml polypropylene centrifuge-tube were placed 250 μl of the fraction to be analyzed, 50 μl of 0.3 *M* magnesium chloride and 50 μl of the pyrophosphatase nucleotide solution. The contents were mixed and after heating for 1 h at 37°C the sample was treated with alkaline phosphatase as described previously. After 25 μl of the internal standard solution and 25 μl of 50% acetic acid were added, 250 μl of each sample were chromatographed using the preparative C_{18} column and the fractions corresponding to solvent front, FUR and FdUR were collected. The fractions were then prepared for determination of radioactivity content as before. The radioactivity profile for the pyrophosphatase plus alkaline phosphatase treated samples is shown in Fig. 5.

Into a 1.5-ml polypropylene centrifuge-tube were placed 100 μl of fraction number 70 (containing FUTP) from the chromatogram of Fig. 1 and the following: 150 μl of Tris–HCl buffer (0.05 *M*, pH 7.8), 25 units UDPG pyrophosphorylase, 100 units inorganic pyrophosphatase and 3.5 mg glucose-1-phosphate. The contents were mixed and allowed to react first at room temperature for 30 min and then at 37°C for an additional 15 min. After the addition of 25 μl ATP solution to serve as a position marker to adjust for small variations in retention times between runs, the mixture was chromatographed using the procedure for the Aminex A-29 column[2]. Fractions were collected and the radioactivity profile determined as shown in Fig. 6.

RESULTS

A typical chromatogram showing the radioactivity profile of the acid-soluble extract obtained when [6-^{3}H]FUra was incubated with S-49 T lymphoma cells is



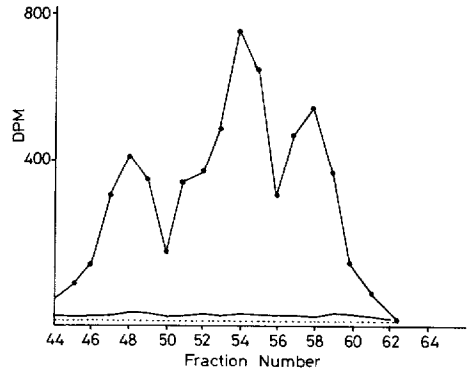


Fig. 4. Preparative reversed-phase chromatogram of fractions 44–62 after treatment with alkaline phosphatase: ———, solvent front; ●—●, FUR; – – –, FdUR.

Fig. 5. Preparative reversed-phase chromatogram of fractions 44–62 after treatment with alkaline phosphatase and pyrophosphatase: ———, solvent front; ●—●, FUR; – – –, FdUR.

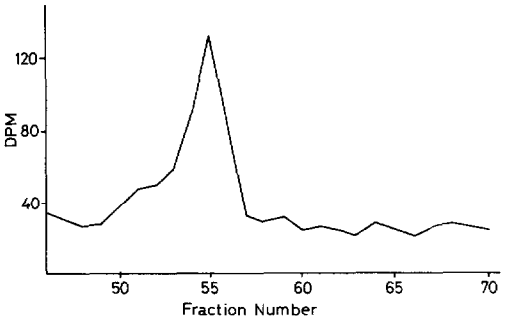


Fig. 6. Aminex A-29 radioactivity chromatogram of [6-^{3}H]FUDP-glucose.

shown in Fig. 1. Fractions 44–62 were analyzed to determine the presence of 5-FUra-nucleotide diphosphosugars. These fractions were treated with alkaline phosphatase which cleaves the phosphate–ribose bond of nucleotides but does not cleave this bond in a nucleotide diphosphosugar. Not all compounds present in these fractions were hydrolyzed as indicated by the presence of a significant amount of radioactivity in the solvent front (Fig. 4). Production of FUR in fractions 56–60 indicated that FUDP was present mainly in these fractions, and the absence of FdUR indicates FdUDP was not present. When fractions 44–62 were treated with pyrophosphatase (which cleaves the phosphate–ribose bond of nucleotide diphosphosugars) and then alkaline phosphatase, radioactivity was found to be absent from the solvent front and present only in FUR as shown in Fig. 5. This indicated the presence of fluorouracil nucleotide diphosphosugars in fractions 46–57. [6-^{3}H]FUTP in fraction 70 from the Aminex A-29 chromatography was used to synthesize [6-^{3}H]FUDP-glucose by a procedure modified from that given for formation of UDP-glucose from UTP[6]. The [6-^{3}H]FUDP-glucose was found to contribute to the radioactivity found in fractions 51–57 as shown in Fig. 6. Since radioactivity in fractions 46–50 is not due to [6-^{3}H]FUDP-glucose, then another FUDP-hexose such as [6-^{3}H]FUDP-galactose is present because it is also hydrolyzed by pyrophosphatase.

In this report, we present a procedure for the determination of FUDP-glucose as a metabolite after fluorouracil is incubated in a suspension of S-49 mouse T-lymphoma cells. Also another FUDP-hexose is shown to be a product of 5-FUra metabolism.

ACKNOWLEDGEMENT

Supported in part by grant CA 27866 from the National Cancer Institute and a Health Education Welfare-Minority Access Research Center Faculty fellowship.

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