Journal of Chromatography, 417 (1987) 57–63
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3616

TECHNIQUE FOR DETECTION OF DNA NUCLEOBASES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OPTIMIZED FOR QUANTITATIVE DETERMINATION OF THYMIDINE SUBSTITUTION BY IODODEOXYURIDINE

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(First received October 21st, 1986; revised manuscript received January 19th, 1987)

SUMMARY

A reversed-phase high-performance liquid chromatographic (HPLC) method for quantification of iododeoxyuridine (IdUrd) substitution for thymidine in DNA was developed. IdUrd substitution was determined by HPLC with ultraviolet absorbance in two cell lines (L1210 and HT-29) after incubation in vitro with IdUrd alone or with IdUrd and fluorodeoxyuridine. In addition, radiolabeled IdUrd was used concomitantly with HPLC to evaluate the degree of dehalogenation. This HPLC technique has also been applied to the measurement of IdUrd incorporation in vivo into DNA from peripheral granulocytes of patients receiving IdUrd. This method provides an interesting tool for the quantification of drug substitution into DNA and can be applied to multiple sampling sites in animal and human studies.

INTRODUCTION

Several drugs (purine and pyrimidine analogues) must be incorporated into DNA in order to produce their effect. They must be transported into cells and undergo metabolic activation within the cells in order to serve as substrates for DNA synthesis. It is useful for both in vitro and in vivo studies to be able to detect and quantify the degree of substitution of these drugs into the DNA.

Quantification of replacement of DNA thymidine (dThd) by halogenated analogues has been reported for a variety of methods. A previously published high-performance liquid chromatographic (HPLC) technique [1] for quantification of bromodeoxyuridine (BrdUrd) substitution used a mobile phase with high salt concentration which requires constant care in order to maintain a reproducible

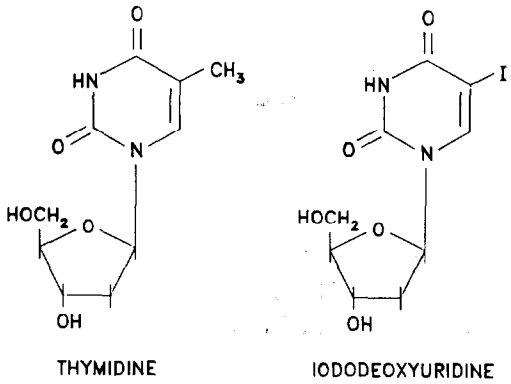

Fig. 1. Structures of thymidine (dThd) and iododeoxyuridine (IdUrd).

system. A monoclonal antibody technique has been used to estimate the proportion of cells which have incorporated iododeoxyuridine (IdUrd) in DNA but this technology has not been applied to quantify dThd replacement in DNA [2]. A gas chromatographic–mass spectrometric (GC–MS) technique has been described for the determination of dThd replacement by BrdUrd [3]. In addition to more expensive hardware, it requires extraction and derivatization to measure by GC–MS and, therefore, it is more labor-intensive. In this paper, a reversed-phase HPLC technique is described for determination of nucleobases that has been optimized for quantification of dThd substitution by IdUrd.

IdUrd is a dThd analogue (Fig. 1) which can sensitize cells to the toxic effect of radiation [4–7]. The radiosensitization effect of this drug is directly proportional to the extent of its incorporation into DNA [8,9].

Fluorodeoxyuridine (FdUrd), a drug which decreases formation of thymidylate by blocking the enzyme thymidylate synthetase [10,11], has often been used in vitro to increase the incorporation of IdUrd into DNA [12,13].

Two different cell lines, HT-29 and L1210, were incubated with IdUrd alone or with a combination of IdUrd and FdUrd. The degree of dThd substitution into DNA of these cells was measured by our technique using HPLC and ultraviolet (UV) absorption and was verified using radioactivity.

In the metabolic process, IdUrd can be dehalogenated inside the cell and subsequently contribute to the endogenous dThd pools [14]. This dehalogenation is catalyzed by the enzyme thymidylate synthetase [15].

Radioactive IdUrd and reversed-phase HPLC separation have been used jointly to determine the degree of this conversion in HT-29 and L1210 cell lines.

EXPERIMENTAL

Drugs and chemicals

Nucleosides (2′-deoxycytidine, dCyd; 2′-deoxyguanosine, dGuo; 2′-deoxyadenosine, dAdo; dThd; IdUrd; FdUrd), enzymes (phosphodiesterase I, type VII, from crotalux atrox venom, and deoxyribonuclease I, DNase I, type II, from bovine pancreas), and DNA (type VIII, from *Escherichia coli*) were purchased from

Sigma (St. Louis, MO, U.S.A.). [6-^{3}H]IdUrd (15 Ci/mmol) was supplied by Moravek Biochemicals (Brea, CA, U.S.A.). McCoy's 5A medium without L-glutamine and Fisher's medium with L-glutamine were purchased from Quality Biologicals (Gaithersburg, MD, U.S.A.). Heat-inactivated fetal bovine serum and trypsin-EDTA were purchased from Gibco Labs. (Grand Island, NY, U.S.A.) and heat-inactivated horse serum from Hazleton Research Products (Denver, PA, U.S.A.). Penicillin G–streptomycin mixture was obtained from Whitaker M.A. Bioproducts (Walkersville, MD, U.S.A.). The organic solvents, methanol and acetonitrile, were HPLC grade purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). The glacial acetic acid was ACS grade from Fisher (Fair Lawn, NJ, U.S.A.).

HPLC conditions

The HPLC mobile phase contained 100 m*M* acetic acid with 4% (v/v) acetonitrile, pH 5.45. The mobile phase was passed through a 0.45-μm filter to remove any particulate matter and to degas the solvent. The solvent was pumped via a Waters Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), isocratically at a flow-rate of 2 ml/min, through a Waters Radial-Pak C_{18} column (10$\times$0.8 cm, 5 μm particle size). The nucleosides were detected simultaneously at 254 nm (Waters Model 440 absorbance detector), 0.1 absorbance units full-scale (a.u.f.s.) and at 300 nm (SF770 detector, Schoeffel Instruments, Westwood, NJ, U.S.A.), 0.01 a.u.f.s. Peak-height ratios at two wavelengths were used to verify the purity of the IdUrd and dThd peaks. The retention times for dCyd, dGuo, dThd, dAdo, and IdUrd were 3.3, 6.3, 7.7, 14.8, and 16.2 min, respectively. It was noted that variations in pH, salt concentration, or percentage of acetonitrile in the mobile phase could produce significant differences in the retention time and order of elution of the nucleosides. Although dAdo and IdUrd eluted close to each other in this system, the minimal absorbance of dAdo at 300 nm facilitated the distinction between these two peaks (Fig. 2).

In the in vitro experiments using [6-^{3}H]IdUrd, hydrolyzed DNA was collected in 1-min fractions for 20 min after injection into the HPLC system. Each fraction was diluted in 10 ml of liquid scintillation counting solution (Hydrofluor, National Diagnostics, Somerville, NJ, U.S.A.) and counted in a Searle Mark III scintillation counter for 10 min.

Cell culture

L1210 cells (suspension culture) were maintained at 37°C in a 5% carbon dioxide atmosphere, in Fisher medium containing 10% heat-inactivated horse serum, penicillin, and streptomycin. The density of L1210 at the start of the incubation was 10^5 cells per ml. Cells were incubated for 24 h (approximately two doublings of untreated cells) without drug, with [6-^{3}H]IdUrd (3 μM, 0.1% hot) alone or simultaneously with [6-^{3}H]IdUrd (3 μM, 0.1% hot) and FdUrd (50 n*M*). At the end of the incubation the cells were counted in a Coulter counter and washed twice with phosphate-buffered saline (PBS) before isolation of DNA.

HT-29 cells (monolayer culture) were maintained at 37°C in a 5% carbon dioxide atmosphere, in McCoy medium containing 10% heat inactivated fetal

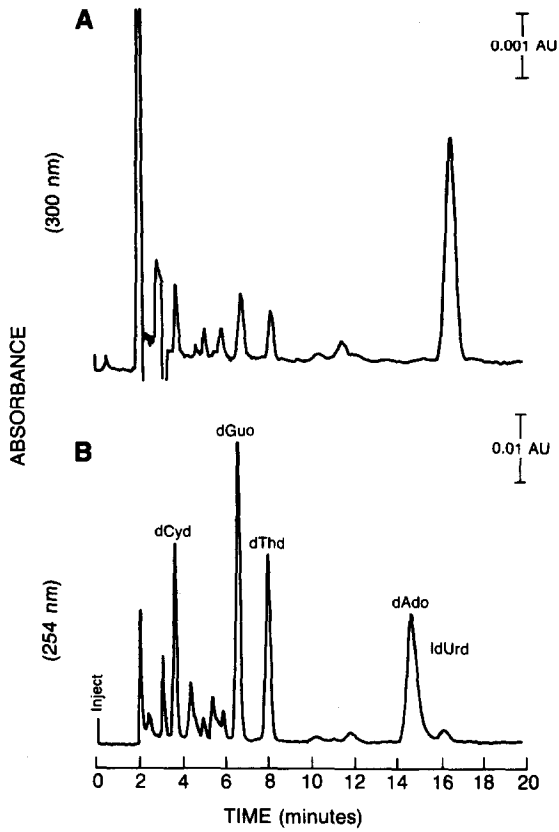

Fig. 2. Chromatograms of DNA extract from L1210 cells ($2 \cdot 10^6$) after incubation with IdUrd. The absorbance was measured simultaneously at (A) 300 nm at an attenuation of 0.01 a.u.f.s. and (B) 254 nm at an attenuation of 0.1 a.u.f.s.

bovine serum, penicillin and streptomycin. Experiments used HT-29 cells at a density of $2 \cdot 10^6$ cells per 75 cm^2 flask, in 15 ml of media. Cells were incubated for 72 h (approximately two doublings of untreated cells) without drug, with [6-^{3}H]IdUrd (3 μM, 0.1% hot) alone or simultaneously with [6-^{3}H]IdUrd (3 μM, 0.1% hot) and FdUrd (50 nM). At the end of the incubation the cells were removed from the side of the flask by trypsin, then counted and washed twice with PBS before isolation of DNA.

DNA extraction and hydrolysis

DNA was isolated using a modification of the technique described by Karle et al. [16]. The cells were disrupted by adding 1 ml of ice water to each pellet ($1 \cdot 10^6$–$5 \cdot 10^6$ cells) and vortexing. Then, the nucleic acids were precipitated by adding 1 ml of 10% trichloroacetic acid (TCA) 0–4 °C and vortexing. After centrifugation at 1000 g for 10 min at 4 °C, the supernatant was discarded. This TCA precipitation was repeated three times. The nucleic acid precipitate was resuspended in 1 ml of alkaline solution (0.25 M sodium hydroxide, pH 11.8) and incubated 90 min at 37 °C for RNA hydrolysis. At the end of this incubation, DNA

was precipitated twice again with cold 10% TCA. The supernatant containing the acid-soluble ribonucleotides was discarded and the DNA precipitate was washed twice with PBS to remove excess TCA.

Each sample of DNA was incubated at 37°C for 10 h with 0.48 U of phosphodiesterase and 140 μg of DNase in 1 ml of 0.05 *M* potassium phosphate buffer, pH 7.45, without EDTA. It was verified that in these conditions, phosphodiesterase, DNase and the contaminant enzymes present in commercial preparations were adequate to break DNA down to nucleosides. *E. coli* DNA (200 μg) was hydrolyzed under the same conditions as control for DNA digestion on each day of analysis. Aliquots (200 μl) of the hydrolyzed DNA (approximately 10–50 μg) were analyzed by HPLC.

Calculations

The method was calibrated based on the absorbance of known concentrations of standard nucleosides analyzed under the same HPLC conditions. IdUrd was determined by absorbance and simultaneously by radioactivity in all studies in vitro.

The ratio (mol dThd + mol IdUrd)/mol dGuo was calculated for each sample of DNA. This ratio was used to verify that the recoveries of IdUrd and dThd were equivalent regardless of the rate of replacement into DNA.

The percentage of substitution was calculted as follows:
mol IdUrd × 100/(mol IdUrd + mol dThd) = percentage of substitution by IdUrd.

The dehalogenation of IdUrd was calculated as follows:
dpm in dThd peak × 100/(dpm in dThd peak + dpm in IdUrd peak) = percentage of IdUrd as dThd into DNA.

RESULTS

In DNA samples isolated from untreated cells (L1210 or HT-29), the IdUrd elution region was free of interfering peaks. In DNA isolated from cells after exposure to IdUrd, a peak with retention time and absorbance ratio corresponding to IdUrd was detected (Fig. 2). When L1210 cells were incubated with IdUrd (3 μ*M*), the dThd substitution by IdUrd was 32%. Of the total amount of drug found in DNA, 11% was incorporated after dehalogenation. When 50 n*M* of FdUrd were added to the media, the dThd substitution increased to 39% while dehalogenation decreased to 8% (Table I). Growth inhibition increased from 8% with IdUrd alone to 24% after addition of FdUrd. This increase in growth inhibition may be due in part to FdUrd toxicity.

For HT-29 cells treated with 3 μ*M* of IdUrd, the dThd substitution by IdUrd was 9% and the incorporation into DNA after dehalogenation of the drug was 26%. When FdUrd (50 n*M*) was added to the media the dThd substitution increased to 14% while the dehalogenation decreased to 18% (Table I). There was a growth inhibition of 50% after incubation with IdUrd alone. No additional short-term toxicity was observed with the addition of FdUrd.

For all studies, the percentage of substitution calculated from absorbance was equivalent to the substitution calculated using radioactivity (Table I). The ratio

TABLE I

THYMIDINE SUBSTITUTION BY IdUrd AS DETERMINED BY RADIOACTIVITY AND ULTRAVIOLET ABSORBANCE

	Growth inhibition (%)	Dehalogenation (%)	Substitution from radioactivity (%)	Substitution from absorbance (%)	(dThd + IdUrd)/dGuo ratio
L1210					
Control	–	–	–	–	1.2
IdUrd	8	11	32	33	1.3
IdUrd + FdUrd	24	8	39	39	1.2
HT-29					
Control	–	–	–	–	1.4
IdUrd	50	26	9	9.8	1.3
IdUrd + FdUrd	50	18	14	14	1.2

(dThd + IdUrd)/dGuo was constant for all levels of substitution.

DISCUSSION

The effects of drugs like IdUrd and other purine and pyrimidine analogues are a consequence of their incorporation into DNA. In experiments designed to investigate various strategies or manipulations of these drugs, it is desirable to measure a direct determinant of drug action.

In these studies, UV absorbance and chromatographic properties of IdUrd and endogenous nucleosides have been used for determination of dThd substitution in L1210 cells and in HT-29 cells. Cells were incubated for approximately two doubling times with external concentrations of IdUrd achievable in humans during a fourteen-day continuous intravenous infusion [17]. When FdUrd was added to the media, the dThd substitution increased 21 and 45% in L1210 cells and HT-29 cells, respectively.

Radiolabeled drugs have often been used to probe biochemical effects of drug administration. The application of this technology for in vivo studies is limited by the total amount of radioactivity which can be administered to human subjects. In addition, for IdUrd which can be dehalogenated inside the cell, counting the total incorporation of radioactivity without separation of nucleosides may lead to improper conclusions [18]. In these in vitro experiments, the continued use of radiolabeled IdUrd and the HPLC method permitted an evaluation of the degree of dehalogenation in these two cell lines, as well as the calculation of the IdUrd substitution into DNA simultaneously by two different techniques.

After counting radioactivity under the dThd peak, it was found in HT-29 and L1210 cells, that 26 and 11% of IdUrd, respectively, was incorporated into DNA as dThd after dehalogenation. Dehalogenation decreased by 27 and 26% in L1210 and HT-29 cells, respectively, when FdUrd was added to the media. These results show that the degree of dehalogenation varies between cell lines. Looking at dThd and IdUrd peaks after HPLC separation, equivalent values for substitution were calculated from UV absorbance and from radioactivity.

At our institution, there is a clinical protocol using IdUrd in patients as a radiosensitizer [2,17,19]. This new technique has been used to quantify the percentage of dThd substitution in vivo in the DNA of circulating granulocytes of patients receiving IdUrd by continuous intravenous infusion [19]. In DNA extracted from $4 \cdot 10^6$ cells, it was possible to measure substitution as low as 0.2%; at the end of the fourteen-day infusion, a substantial degree of substitution (7–17%) was observed.

In conclusion, the described HPLC technique is simple and sensitive enough for quantification of dThd substitution by IdUrd in vivo and in vitro without use of radioactivity. By modification of HPLC conditions, this technique may be optimized for measurement of DNA incorporation of other purine and pyrimidine analogues. In addition, with appropriate sample preparation, this method can be applied to multiple sampling sites (tumor or normal tissue biopsies) in animal and human studies. This will facilitate the correlation of routes, schedules, and combinations of drug administration with appropriate target effects, i.e., purine or pyrimidine replacement in tumor and normal tissues.

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