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ANALYSIS OF 5-FLUORO-2′-DEOXYCYTIDINE AND 5-TRIFLUOROMETHYL-2′-DEOXYCYTIDINE AND THEIR RELATED ANTIMETABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An isocratic, ion-paired, reversed-phase high-performance liquid chromatography technique for the quantitative determination of 5-fluoro-2′-deoxycytidine (FdCyd) and 5-trifluoromethyl-2′-deoxycytidine (F_3methyldCyd) and their related antimetabolites is described. Extraction and purification of these compounds from DNA, RNA, and free pools is reviewed diagrammatically. Total analysis time including quantitation of DNA and RNA primary constituents is 45 min. Average combined recoveries for prodrugs and antimetabolites is above 90% with standard deviations of 0.07 and 0.58 and average precisions of 5.51 and 8.30% for FdCyd and F_3methyldCyd, respectively. Average coefficients of variation were 3.8 ± 0.5% for FdCyd and 7.7 ± 1.0% for F_3methyldCyd. Limits of detection were approximately 1 pmol for unlabelled prodrugs and antimetabolites. FdCyd, when

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generally tritiated with a specific activity of 18 Ci/mmol, was detected in the $5 \cdot 10^{-15}$—$20 \cdot 10^{-15}$ mol (fmol) range depending on sample condition.

INTRODUCTION

Fluoropyrimidines are widely used in the treatment of neoplastic disease. One of the major drugs utilized in the prevention of metastatic disease and against malignancies of the breast and gastrointestinal tract is 5-fluorouracil (FUra) [1, 2]. Although widely used both as a single agent and in combination chemotherapy, FUra possesses several shortcomings as an antineoplastic agent [3—5]. These deficiencies are a consequence of the susceptibility of FUra to systemic catabolism is its general lack of antineoplastic selectivity which commonly results in bone marrow and intestinal toxicities [6—8].

Early research by Heidelberger et al. [7] utilized Dowex® chromatographic gels for the separation and quantitation of metabolites following exposure to mammalian cells, a separatory technique incapable of fully resolving all the metabolic products formed during FUra exposure. These studies also failed to detect FUra antimetabolites in the DNA of mammalian cells [9—11]. Fine-tuned high-performance liquid chromatographic (HPLC) procedures coupled with vastly improved synthesis techniques, yielding greater specific activities in labeled compounds, have permitted the quantitation of FUra metabolic products incorporated into mammalian DNA in cell culture [12, 13] and more recently in animal tissue [14—16].

5-Trifluoro-2′-deoxythymidine (F_3dThd), which was first synthesized in the laboratory of Heidelberger (who also first synthesized FUra), has had limited use as an antitumor agent owing to its rapid catabolism and general lack of selectivity [17]. Recently, HPLC methodologies have been described for the separation of F_3dThd from its catabolic derivatives, 5-trifluorothymine and 5-carboxyuracil, in herpes keratitis [18].

To address the problems of catabolism and lack of selectivity of FUra and F_3dThd, our laboratory has developed two new chemotherapeutic approaches, which circumvent catabolism and result in preferential toxicity to tumor cells. 5-Fluoro-2′-deoxycytidine (FdCyd) and 5-trifluoromethyl-2′-deoxycytidine (F_3methyldCyd), when coadministered with tetrahydrouridine (H_4Urd), a potent inhibitor of cytidine deaminase (CD), have been shown to be efficacious against Lewis lung carcinoma (LLC) and mammary adenocarcinoma-755 (ADC-755) [19, 20].

To establish the biochemical basis for the increased efficacy of FdCyd or F_3methyldCyd with H_4Urd compared to that of 5-fluoro-2′-deoxyuridine (FdUrd), F_3dThd, FUra or FdCyd and F_3methyldCyd administered alone [19, 21], HPLC methodologies were developed for the quantitation of antimetabolites formed in tumor and normal tissue and their incorporation into DNA and RNA.

Procedures directed towards the analysis of fluoropyrimidines possessing structures similar to FdCyd and F_3methyldCyd have in the past employed buffer gradients to resolve antimetabolites from normal constituents of DNA and RNA [11, 14]. While these methods were sensitive and accurate, they permitted quantitation of the compounds of interest only by scintillation counting due to

the baseline aberrations created by gradient elution. The purpose of this study was two-fold. (i) To develop an accurate isocratic HPLC method for the quanti tion of FdCyd- and F_3methyldCyd-related antimetabolites incorporated into DNA and RNA as well as the quantitation of antimetabolite pools. (ii) To refine this method for the quantitation of unlabeled antimetabolites and their resolution from normal constituents of DNA and RNA.

The procedures outlined in this paper permit extraction (see Figs. 1 and 2) and quantitative analyses of the normal and antimetabolite constituents of DNA and antimetabolite pools.

EXPERIMENTAL

Apparatus

A liquid chromatograph equipped with a Model U6K universal injector, a Model 6000A solvent delivery system attached to a Model 450 variable-wavelength detector, two Model 440 fixed-wavelength detectors (Waters Assoc., Milford, MA, U.S.A.) and two Model LD11B strip-chart recorders (Westronics, Fort Wayne, TX, U.S.A.) was utilized. HPLC integration was accomplished with a Model 3390 reporting integrator (Hewlett-Packard, Deerfield, IL, U.S.A.). The HPLC columns used were a Bondapak C_{18} (5 μm bead size, Waters Assoc.) and an Alltech 600 RP-C_{18} (10 μm bead size, 250 × 4.6 mm I.D., Alltech Assoc.). The syringe was a 10-μl Model 820 (Hamilton, Reno, NY, U.S.A.).

Recorder modifications. To the Westronics Model LD11B strip-chart recorders were affixed 1-mV range resistors which increased the signal-to-noise ratio by a factor of 10, permitting attenuations of 0.0002 absorbance units full scale (a.u.f.s.).

Reagents for HPLC studies

Tetrabutylammonium phosphate (Eastman Kodak, Rochester, NY, U.S.A.) and dibasic ammonium phosphate (Mallinckrodt, St. Louis, MO, U.S.A.) were used as a primary elution buffer. All buffers were filtered through 0.45-μm Millipore filters (Bedford, MA, U.S.A.) under reduced pressure and buffer solutions were always degassed prior to use. Nanograde® methanol was obtained from Mallinckrodt; all antimetabolite standards, including FdCyd and F_3methyldCyd, were prepared separately in deionized water at a 10 m*M* concentration.

Synthesis of nucleotide analogues

Synthesis of the monophosphate analogues of FdCyd and F_3methyldCyd was accomplished using the method of Tanaka et al. [22]. Briefly, 90 mg of the deoxycytidine analogues were separately reacted with phosphorous oxychloride (0.55 m*M*) in the presence of triethyl phosphate (1.5 ml). After extraction with two chloroform and six water washings (2 ml each), the aqueous layers were combined and neutralized with sodium bicarbonate, and diluted with deionized water (100 ml). Prior to sample application, a DEAE-cellulose column (bicarbonate, 26 cm × 3 cm) was washed with 1 l of deionized water and the sample was eluted with a linear gradient of 0 to

400 m*M* triethylammonium bicarbonate; this buffer was produced by bubbling carbon dioxide into a 0.4 *M* solution of triethylamine while stirring until a pH of 5.0 was attained. The monophosphate was detected by absorbance at 280 nm.

Reference standard and response calibration

Occasionally, the concentrations of certain metabolites of interest were difficult to quantify in their untritiated form. In these instances, between 1 and 10 pmol of that standard compound were added to the sample and a second analysis was performed. This procedure facilitated measurement of the recorder response obtained for each compound and allowed differential quantitation of the material.

Calculations

Quantitation of each compound of interest when untritiated (with a detection limit of 1.0—2.0 pmol) was accomplished by measuring recorder responses and following the peak comparison, external standard method described by Ceri [21]. Quantitation at the fmol level was accomplished by analysis of tritiated FdCyd and F_3methyldCyd metabolites via scintillation counting.

Reproducibility of standards

The reproducibility of HPLC analysis was determined by injecting aliquots of standard solutions of all compounds of interest including the analogues, FdCyd or F_3methyldCyd. Recorder sensitivity was adjusted for each standard to 50% recorder response at 0.001 a.u.f.s. The resulting peak heights varied from 134 to 139 mm with an average variance of 0.3 mm and a standard deviation of 0.6 mm. Each standard was injected a minimum of five times.

Extraction efficiency

Medium samples were spiked with known amounts of reference standards. The samples were extracted as outlined below. Recovery data obtained from spiking ten 5-ml samples indicated a recovery (mean ± S.D.) of 91 ± 0.5% for the pyrimidine analogues and a coefficient of variation (C.V.) of 7.0%. The same procedure was used for assessing extraction of normal deoxynucleoside and deoxynucleotide components of DNA. No loss of greater than 10% was detected using the extraction procedure outlined in Fig. 2.

Buffer solutions

The mobile phase consisted of 0.005 *M* tetrabutylammonium phosphate—0.0025 *M* dibasic ammonium phosphate, pH 6.8. To this basic solution were added varying amounts of methanol. Following extraction of samples as described diagrammatically (Figs. 1 and 2), frozen aliquots were lyophilized and reconstituted in 1.0 ml of deionized water and 25 μl were injected into a pre-equilibrated HPLC column.

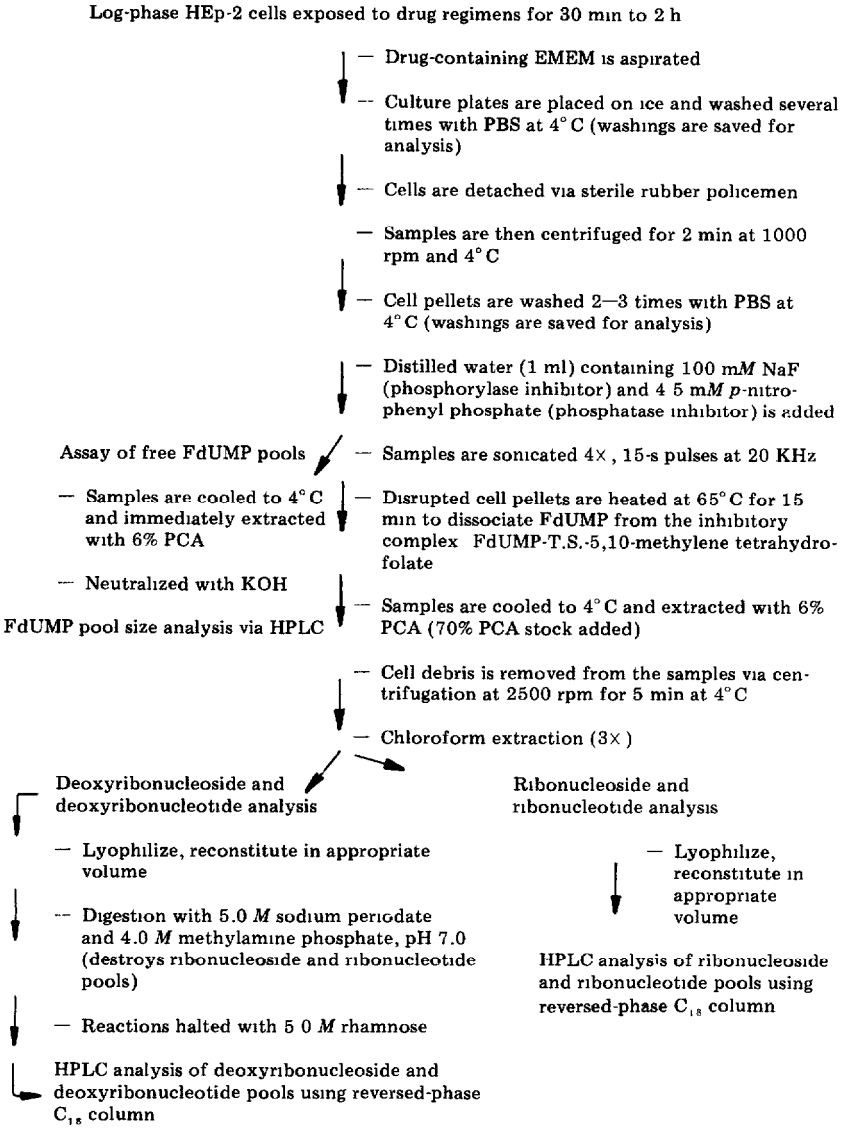

Fig. 1. Pool size extraction procedure which is described in detail by Boothman et al. [12] and applicable to in vivo and in vitro extractions and purifications. HPLC parameters described briefly in the Experimental section were as follows. Column, Alltech High Load RP-600 reversed-phase C_{18} column; paired-ion buffer, 0.005 *M* tetrabutylammonium phosphate—0.005 *M* dibasic ammonium phosphate, pH 6.80 plus Nanograde methanol (90 ml/l buffer); flow-rate, 0.7 ml/min (47 bar); detector sensitivity, 0.0002 a.u.f.s. Abbreviations used: EMEM = Eagle's minimal essential medium; KHz = kilohertz; PBS = phosphate-buffered saline; PCA = perchloric acid; TS = thymidylate synthetase.

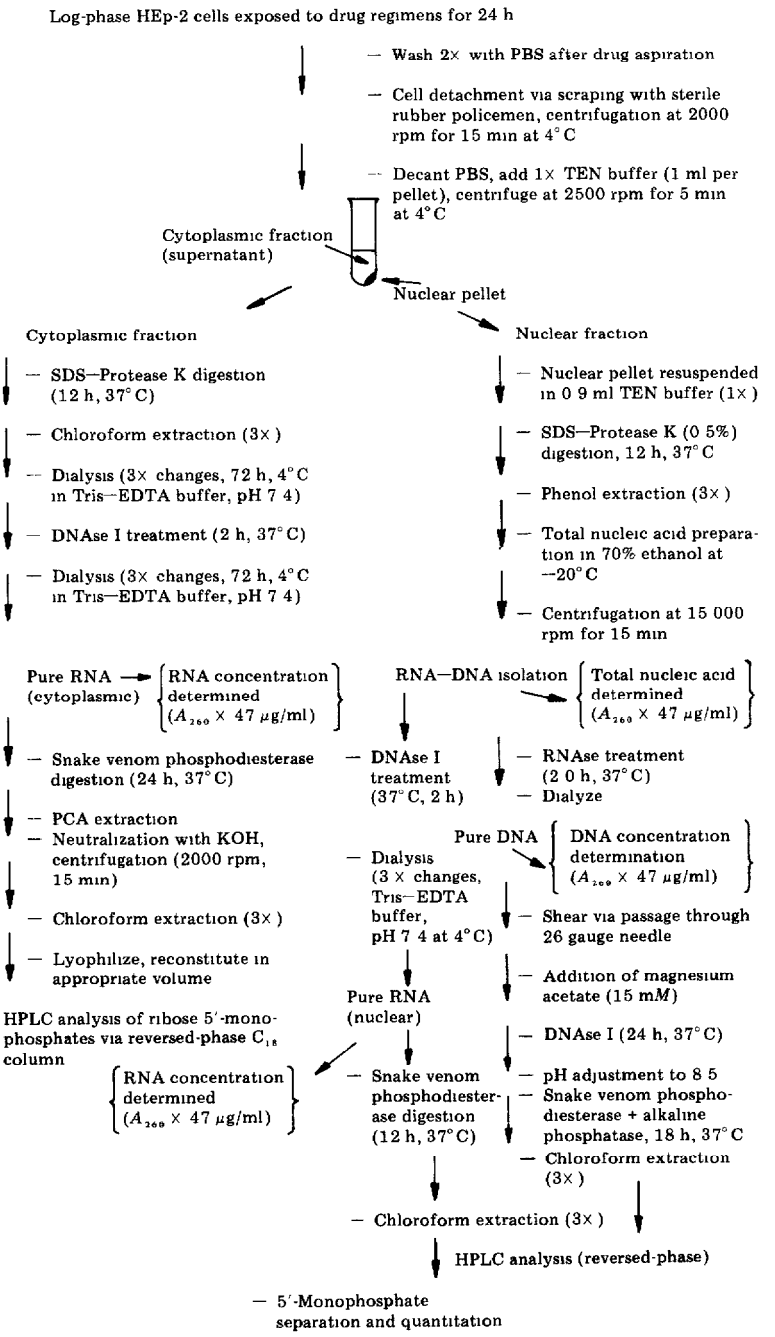

Fig. 2. Comprehensive flow chart of the wet chemistry extraction and purification procedure utilized in vivo or in vitro for the analysis of normal DNA and RNA and antimetabolite pool size constituents. This method and the subsequently described HPLC analysis may be used for both nuclear and cytoplasmic RNAs. Centrifugations indicated in Figs. 1 and 2 utilized a Sorvall RC-2 instrument with an HB-4 rotor (Newtown, CT, U.S.A.). Abbreviations used: EDTA = ethylenediaminetetraacetic acid; TEN = 10 m*M* Tris pH 8.0, 100 m*M* sodium chloride and 1.0 m*M* Na_2EDTA; SDS = sodium dodecyl sulfate.

RESULTS

Initially, gradient elution techniques were employed to resolve FdCyd and F_3methyldCyd and their related antimetabolites from normal constituents of DNA. Methodology previously reported [11, 14] utilizing similar paired-ion chromatographic (PIC) buffers was employed with little success, due to the erratic baseline slopes encountered when using high detector sensitivities. While these systems were efficient and accurate with radiolabeled metabolites, they did not permit quantitation by peak-height measurement. To circumvent these problems, various concentrations of methanol were added to the basic PIC buffer as needed to resolve particular metabolites of interest. Quantitation of antimetabolites and unreacted drugs was conducted by isocratic HPLC analysis using the above described buffer with 9% methanol at a flow-rate of 1.0 ml/min and a detector sensitivity of 0.0002 a.u.f.s. The above-mentioned, parameters provided sharp, single elution responses for FUra, FdCyd, 5-fluorouridine (FUrd), FdUrd, 5-fluoro-2′-deoxycytidine-5′-monophosphate (FdCMP) and 5-fluorouridine-5′-monophosphate (FUMP) (see Fig. 4). These elution conditions were also compatible for the analysis of F_3methyldCyd and F_3dThd (see Fig. 6).

Due to the chromatographic properties of certain phosphorylated antimetabolites, a second analytical procedure utilizing HPLC was performed to achieve better separation of the antimetabolites mentioned below. Quantitation of 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), 5-trifluoromethyl-2′-deoxycytidine-5′-monophosphate (F_3methyldCMP) and 5-trifluoro-2′-deoxythymidine-5′-monophosphate (F_3dTMP) was accomplished by isocratic analysis with a mobile phase, as described previously, employing a 20% methanol concentration at a flow-rate of 1.5 ml/min and a detector sensitivity of 0.0002 a.u.f.s. This system permits appropriate resolution of the previously mentioned metabolites while providing elution times under 30 min.

HPLC analysis for the determination of FdCyd FdUrd, F_3methyldCyd or F_3dThd incorporated into DNA

Following extraction, purification and enzymatic digestion (illustrated in Fig. 1), the frozen DNA samples were thawed and dried under nitrogen purge. After reconstitution in 1.0 ml of deionized water, 25-μl aliquots were injected into a pre-equilibrated high-performance liquid chromatograph column and analyzed using 9% methanol in the mobile phase at a flow-rate of 0.7 ml/min and a detector sensitivity of 0.0002 a.u.f.s.

The above-mentioned conditions provided analytical techniques which were compatible for the analysis of FdCyd, FdUrd or F_3methyldCyd and F_3dThd incorporated into DNA, as well as for the quantitation of the normal constituents of DNA. Integrator settings for this and all mentioned analyses were as follows: threshold, 1.0; attenuation, 0.0; peak height, 0.64; chart speed, 0.2 cm/min; baseline, variable.

Integrator and strip-chart recorders for both 254- and 280-nm detectors were started simultaneously upon sample injection. The retention times, shown on the chromatographic profile of HPLC analysis of DNA were recorded using samples spiked with 5—10 pmol of FdUrd, FdCyd or F_3methyldCyd, F_3dThd standards and 10—20 pmol of normal deoxyribonucleoside standards.

Quantitation of antimetabolites incorporated into RNA and antimetabolite pool size evaluations

Due to the chromatographic differences between normal RNA nucleoside constituents and FdCyd-related antimetabolites, the extraction and purification of FdCyd-related antimetabolites incorporated into RNA were performed using the protocol illustrated in Fig. 2. Additionally, quantitation of antimetabolite pool size levels was accomplished by using the protocol illustrated in Fig. 1. The following parameters were employed for the quantitation of DNA-related metabolites listed in Fig. 1, antimetabolites incorporated into RNA and antimetabolite pools. Column, Alltech RP 600 C_{18} reversed-phase; buffer, 0.005 *M* tetrabutylammonium phosphate—0.005 *M* dibasic ammonium phosphate; pH, 6.80 + 9% methanol; flow-rate, 1.0 ml/min (80—100 bar); detector sensitivity, 0.0002 a.u.f.s.

5′-Monophosphate derivatives of the nucleosides of FdCyd, FdUrd, F_3methyldCyd and F_3dThd were prepared [22] and their nucleotides FdCMP, FdUMP, F_3methyldCMP and F_3dTMP were produced with yields of 55, 40, 33 and 21%, respectively.

DNA extraction and purification were initially performed using human laryngeal epidermoid carcinoma (HEp-2) cells in culture and BDF_1 mouse tissues from spleen, liver, intestine, and Lewis lung carcinoma. Duplicates were repeated on four separate DNA analyses with a mean coefficient of variation of 4.3 ± 0.5% (range 1.0—7.3%). Average retention times for normal DNA constituents, as well as antimetabolites incorporated into DNA, are shown in Fig. 3.

Due to the high specific acitivity of tritiated FdCyd (18 Ci/μmol) and the very low detection limits provided by this method, less than 1.0 μg of DNA or $1 \cdot 10^5$ tumor cells was needed to identify FdCyd and its antimetabolites. As such, each analysis was run with a full complement of authentic external

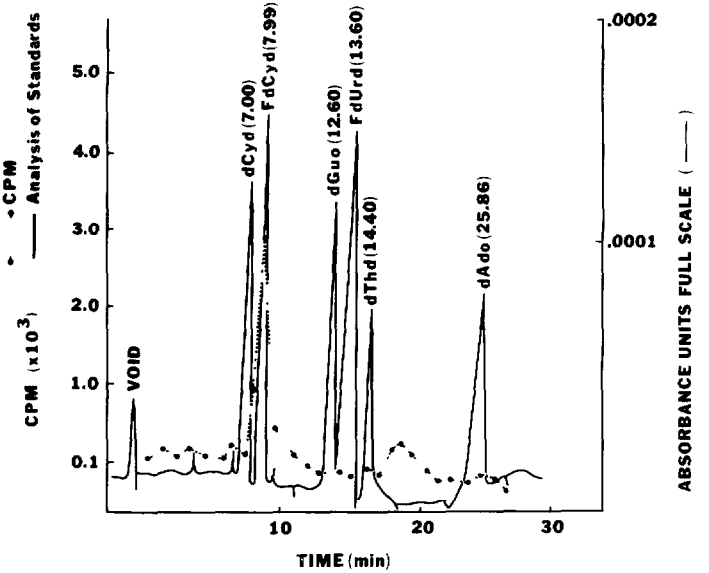

Fig. 3. HPLC analyses of HEp-2 DNA, obtained as described in Fig. 4, from cells treated with FdCyd (0.1 μM) + H_4Urd (2.0 m*M*).

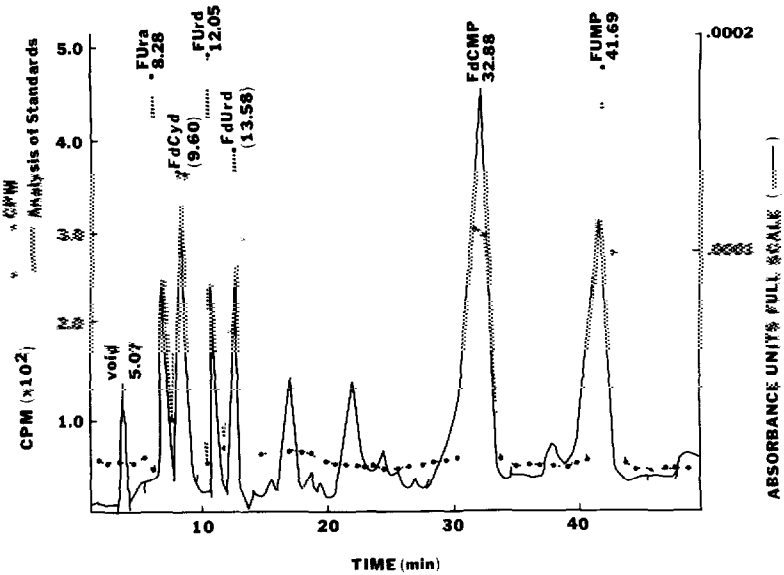

Fig. 4. Chromatogram of an extraction and purification followed by HPLC analysis of prodrugs and antimetabolites using the procedure shown in Fig. 1. HEp-2 cells were utilized for this experiment and authentic standards of the antimetabolites were added to identify compounds of interest and coordinate superpositioning of the radiogram and the chromatogram.

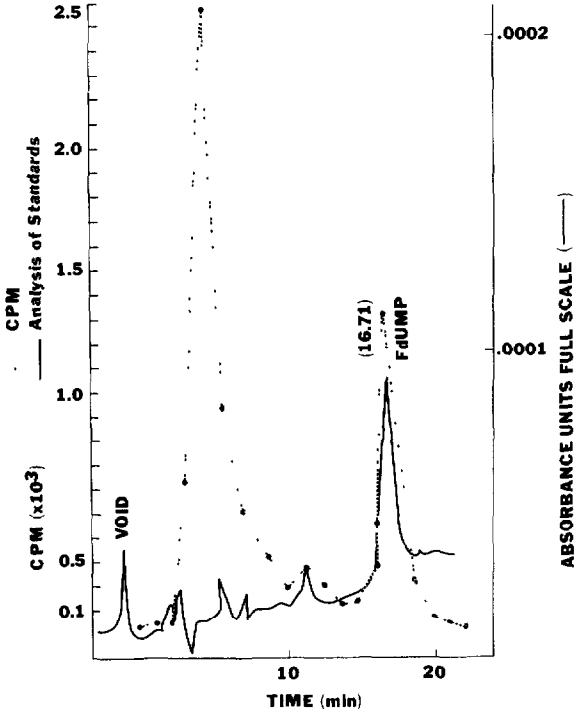

Fig. 5. Isocratic HPLC separation and quantitation of the FdUMP pool size in HEp-2 cells treated with FdCyd (0.1 μM) + H_4Urd (1.0 mM).

standards to facilitate identification of the metabolite. Use of microbore tubing (I.D. 0.0028 mm, Waters Assoc.) virtually eliminated lag time between response detection and collection of the radioactive effluent representative of that response.

Analysis of antimetabolite pools in tissue culture was performed using HEp-2 cells and employing a wide range of FdCyd concentrations. Fig. 4 shows a typical chromatogram with a superimposed radiogram of FdCyd and its related antimetabolites. The method described for antimetabolite pool analysis can be utilized for cells in culture as well as for animal tissues. Fig. 5 shows a separation of FdUMP (with a superimposed radiogram), an important metabolite of FdCyd which is responsible for the inhibition of thymidylate synthetase, a target enzyme in pyrimidine chemotherapy. Long retention times for FdUMP ands F_3dTMP (the key inhibitor of thymidylate synthetase derived from F_3methyldCyd) necessitated a separate analysis which also facilitated the quantitation of enzyme-bound (FdUMP-thymidylate-synthetase-5,10-methylene tetrahydrofolate) and unbound (or free) FdUMP levels. Resolution of F_3-methyldCyd and F_3dThd and their separation from DNA nucleosides is shown in Fig. 6; resolution of F_3methyldCMP and F_3dTMP and their separation from DNA nucleotides is shown in Fig. 7.

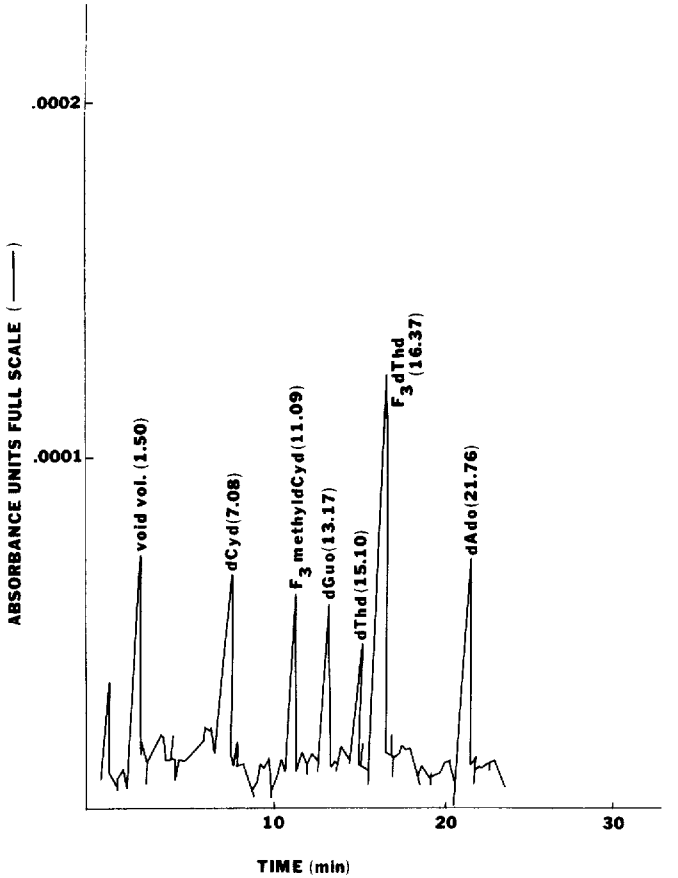

Fig. 6. HPLC separation of F_3methyldCyd, F_3dThd and DNA deoxyribonucleosides.

F_3methyldCyd was not deaminated to F_3dThd (<0.001%) after exposure to 10% fetal calf serum or phosphate-buffered saline (PBS) (no cells added) for 2 h at 37°C. Furthermore, heating for 15 min at 65°C in 10% fetal calf serum or PBS did not result in deamination. Spiked samples were analyzed after extraction via the protocol shown in Fig. 1. Mean recovery as determined with nine samples was 89% with a standard deviation of 7.0% and a coefficient of variation of 4.3 ± 0.7%. Mean recovery for FdCyd after extraction was 92% with a standard deviation of 10.9% and a coefficient of variation of 10.6 ± 1.2%. No deamination (< 0.001%) could be detected with FdCyd under the conditions summarized in Fig. 1 in which the strategy was to avoid heating in acid — a precaution that is not necessary when FUra or FdUrd and their metabolites are analyzed.

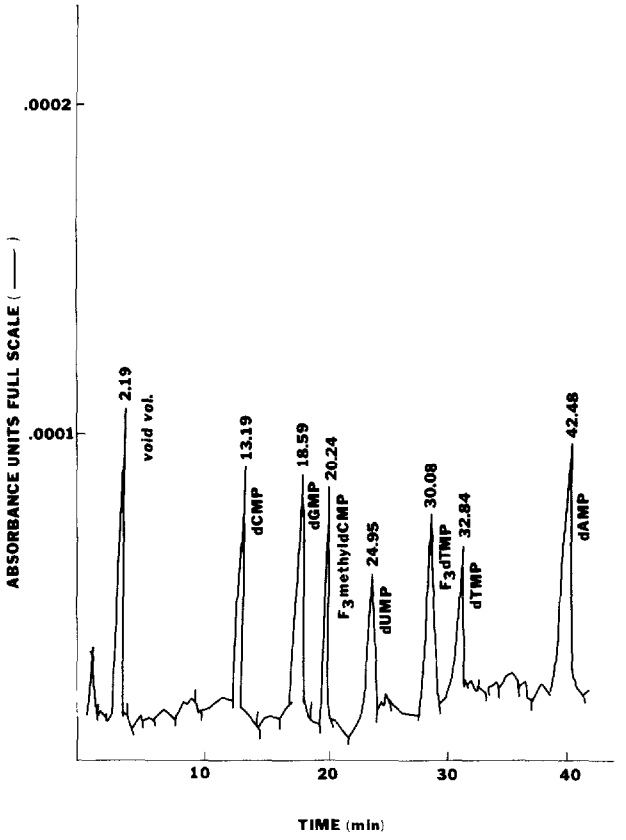

Fig. 7. HPLC separation of F_3methyldCMP, F_3dTMP and DNA deoxyribonucleotides.

DISCUSSION

Previous studies in our laboratory using LLC in BDF_1 mice have indicated that FdCyd [19] and F_3methyldCyd [21], when each was separately coadministered with H_4Urd, displayed greater metabolic stability and antitumor selectivity than FdUrd, FUra or F_3dThd. Recently, we have documented the

channeling of FdCyd towards an exclusively DNA directed effect and the overall advantages obtained when FdCyd is administered with an inhibitor of its deamination [12].

The methodology described in this paper was utilized to add support to our contentions that: (i) FdCyd, when co-administered with an inhibitor of its deamination, is rapidly metabolized to FdCMP and acts as a storage form for the production of the potent thymidylate synthetase inhibitor, FdUMP; (ii) FdCyd forms substantially more FdUMP when co-administered with H_4Urd than will FdUrd at equitoxic doses; and (iii) FdCyd, when metabolically directed by co-administration with H_4Urd, produces little, if any, RNA-level antimetabolites, which may contribute to general toxicity [23, 24]. Analogous proposals have been made for F_3methyldCyd, which inherently lacks an RNA-directed component. Therefore, a rapid isocratic ion-pair HPLC method has been developed for the separation and quantitation of FdCyd, F_3methyldCyd and their related antimetabolites. Due to the unavailability of a commercial source of di- and triphosphate antimetabolites and the relatively lower expense of purified-enzyme preparations, antimetabolite analyses of RNA and DNA were carried out at the nucleoside and nucleotide (monophosphate) levels, respectively (see Fig. 1). Figs. 1 and 2 describing the protocols for 2-h exposure, pool size analysis and DNA/RNA incorporation studies have been thoroughly tested in tissue culture [12] and recently in animals (unpublished data). A few precautions concerning these protocols should be noted. (i) Chloroform extraction should be continued until the interface is completely free of all proteinaceous material because even a small amount of residual protein tends to destroy the guard columns and impair the analysis. (ii) Exhaustive dialysis using Tris buffer, pH 8.0, was also necessary and greatly enhanced enzymatic degradations of DNA and RNA prior to HPLC analysis by eliminating all phenol and phenolic degradation products, as well as restoring the pH to optimize enzymatic activity levels (Fig. 2). As shown by the chromatograms given in this paper, baseline aberrations due to gradient elution, buffer changes and buffer change—sample co-elution have been eliminated. In-laboratory buffer preparation has lowered the cost per analysis over already prepared PIC buffers approximately ten-fold, as has the use of dual-guard columns. Twin Co:Pel ODS (C_{18}) guard columns (Waters Assoc.) have extended the life of a single analytical column to over 1.5 years with almost continuous use. (Guard columns were replaced when operating pressure exceeded 33 bar above normal.)

Prior to each analysis, sample volumes were normalized and a 50-μl aliquot of each radioactive sample was counted and compared to total counts from each DNA, RNA or antimetabolite pool analysis. Statistical evaluation (data not shown) revealed that all counts in 50 μl (> 93%) of each unanalyzed sample were accounted for only in antimetabolite constituents of DNA, RNA or pools. This was particularly important for two reasons: (i) generally labeled FdCyd was shown to be stable under all extraction conditions; (ii) this stability greatly increased our confidence that general exchange with solvent or tritiated exchange of drugs with normal constituents was not occurring during experimentation in vivo or in vitro, thus influencing the quantitation of antimetabolites.

The nature of FdCyd and F_3methyldCyd extraction and purification from DNA, RNA and normal pools lends itself to segmental analyses of all related antimetabolites. Although some other facets of FdCyd and F_3 methyldCyd metabolism have yet to be determined, it was our intention to develop a method which would provide a rapid, quantitative and unobtrusive analysis of the prodrugs and their antimetabolites. The previously described methods [12] shown in Figs. 1 and 2 along with the described HPLC parameters permit accurate analysis of all antimetabolites derived from FdCyd or F_3 methyldCyd. The effect of these two treatments on normal constituents of DNA, RNA, and natural pools in vitro are presently underway, as are animal studies employing these nucleoside analogue treatments, FdCyd (or F_3methyldCyd) + H_4Urd. The methods described here should provide a means by which levels of antimetabolites derived from FdCyd or F_3 methyldCyd can be quantified in tumor and normal tissues, facilitating the elucidation of the routes of conversion and anabolism of the administered nucleoside analogues in serum, normal and tumor tissues. These studies should enable correlations between efficacy and the selective formation of antimetabolites, which should preferentially inhibit target enzymes in neoplastic tissue.

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